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THE UNIVERSITY OF ALBERTA

# EFFECTS OF AGE AND SEX ON INTRAMUSCULAR AND EPIMYSIAL COMPONENTS OF BOVINE TRICEPS BRACHII

by

Takuo Nakano ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

ANIMAL BIOCHEMISTRY

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA

Fall, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Effects of Age and Sex on Intramuscular and Epimysial Components of Bovine Triceps Brachii", submitted by Takuo Nakano in partial fulfilment of the requirements for the degree of Master of Science in Animal Biochemistry.

J R Thompson
Supervisor

R J Berg

G W Mathison

C H Pearson

Date June 17 1976

## ABSTRACT

The study was designed to determine the effects of age, and to a limited extent sex on the bovine triceps brachii intramuscular and epimysial connective tissue components: collagen, elastin, reticulin, glycosaminoglycans, glycopeptides (glycoproteins), and sialic acid. Emphasis was placed on glycosaminoglycan analyses.

The animals used were: 2 groups of fetuses (3 to 5 and 6 to 8 months old), female yearlings (1.2 years old), young male adults (1.6 to 2.0 years old), young female adults (1.7 to 2.1 years old), and mature female adults (7.1 to 8.9 years old). Histochemical analyses were followed by scoring for the connective tissue components. Chemical analyses were conducted on all except fetal expimysial tissue. Chemical data were expressed on a lyophilized-defatted basis.

Histochemically, the fetal groups showed a significantly ($P < 0.05$) higher intramuscular collagen score, a significantly lower epimysial collagen score, and a significantly higher intramuscular and epimysial score of reticulin and mucopolysaccharides than did the post natal groups. Both intramuscular and epimysial reticulin scores were significantly higher in the yearling group than in the adult groups. The mature adult epimysium showed a significantly decreased mucopolysaccharide score : collagen score ratio.

Fetal intramuscular tissue characteristically showed a significantly increased nitrogen concentration and a significantly decreased collagen concentration during the fetal growth period. In post natal groups epimysial collagen concentration increased with age. The highest concentrations were observed in the mature adult and young

male adult groups.

The percentage labile collagen decreased significantly with advancing animal age in both intramuscular and epimysial tissues. The concentrations of intramuscular labile hexosamine, sialic acid and nonscleroprotein nitrogen decreased significantly during fetal growth and were higher in the fetal than in the post natal groups. Epimysial labile hexosamine concentrations were significantly lower in the young male and mature adult than in the yearling and young female adult groups. The mature adult group demonstrated a significantly lower epimysial nonscleroprotein nitrogen concentration than the other post natal groups.

Hyaluronic acid and dermatan sulfate were the major glycosaminoglycans in both the intramuscular and epimysial tissues in all groups. The concentration of hyaluronic acid was approximately twice that of dermatan sulfate in the intramuscular tissue, and approximately 60% that of dermatan sulfate in the epimysial tissue. The mature adult epimysium contained almost equal concentrations of hyaluronic acid and dermatan sulfate. Relatively small amounts of heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and heparin were found in all tissues.

The concentrations of intramuscular hyaluronic acid and dermatan sulfate decreased significantly during fetal growth and were significantly higher in the fetal than in the post natal groups. The mature adult epimysium had the lowest concentration of dermatan sulfate. The age related decrease of the total glycosaminoglycan concentration was associated with a decreased total glycosaminoglycan : collagen ratio.

The concentration of both the glycopeptide fraction and sialic acid were found to decrease with increasing animal age.

These observations indicate that maturation of skeletal muscle connective tissue results in an increased stability of collagen and a decreased relative amount of glycosaminoglycans and glycoproteins.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| BF | Biceps Femoris |
| Ch | Chondroitin |
| Ch4-S | Chondroitin 4-sulfate |
| Ch6-S | Chondroitin 6-sulfate |
| ChS | Chondroitin Sulfate |
| CPC | Cetylpyridinium Chloride |
| CTAB | Cetyltrimethyl Ammonium Bromide |
| ΔDi-OS | 2-Acetamido-2deoxy-3-O-(β-D-gluco-4-enepyranosyl uronic acid)-D-galactose |
| ΔDi-4S | 2-Acetamido-2deoxy-3-O-(β-D-gluco-4-enepyranosyl uronic acid)-4-O-sulfo-D-galactose |
| ΔDi-6S | 2-Acetamido-2deoxy-3-O-(β-D-gluco-4-enepyranosly uronic acid)-6-O-sulfo-D-galactose |
| DS | Dermatan Sulfate |
| GAG | Glycosaminoglycan |
| Gal | Galactose |
| GalN | Galactosamine |
| GlcN | Glucosamine |
| GlcUA | Glucuronic Acid |
| HA | Hyaluronic Acid |
| HP | Heparin |
| HS | Heparan Sulfate |
| IdUA | Iduronic Acid |
| IMCT | Intramuscular Connective Tissue |
| KS | Keratan Sulfate |
| LD | Longissimus Dorsi |
| MPS | Mucopolysaccharides |
| N | Nitrogen |
| RF | Rectus Femoris |
| S.D. | Standard Deviation |
| SM | Semimembranosus |
| SNSPN | Soluble Nonscleroprotein Nitrogen |
| ST | Semitendinosus |
| TB | Triceps Brachii |
| TCA | Trichloroacetic Acid |
| UDP | Uridinediphosphate |

# INTRODUCTION

The understanding of age and sex associated changes in skeletal muscle connective tissue, located in the epimysium, perimysium and endomysium, is of importance in the study of muscle physiology as well as in understanding changes in meat quality during the growth and maturation of the meat producing animal.

The major components in the connective tissue are generally considered to be collagen, elastin and reticulin as the fibrous components, and the glycosaminoglycans and glycopeptides as components of the amorphous ground substance.

Considerable research has been reported on age associated changes in collagen as a factor related to meat tenderness (Goll et al., 1963; Hill, 1966; Shimokomaki et al., 1972; Cross et al., 1973). Elastin has been less extensively studied perhaps because it is a relatively minor component of muscle and because of the intrinsic difficulty to analyse this hydrophobic protein. Quantitative analysis of muscle reticulin has not been reported at this time.

Limited research has been conducted on glycosaminoglycans and glycoproteins in muscle. Glycosaminoglycans are a major component of ground substance and are believed to participate in important in vivo functions such as water retention, electrolyte control, membrane permeability, collagen organization, and clearing activity. Glycosaminoglycans in muscle from meat producing animals have been

investigated (McIntosh, 1966, 1967; Fox, 1968; Wipf, 1969; Wipf et al., 1970; Cormier et al., 1971; Gilbreath et al., 1971), however there is a need for more definitive information on the quantity and types of muscle glycosaminoglycans.

The purpose of this investigation was to study the age and sex related changing pattern in a variety of connective tissue components; collagen, elastin, reticulin, glycosaminoglycans, glycopeptide and sialic acid in muscle. Considerable emphasis was placed on the development of analytical techniques for the determination and characterization of muscle glycosaminoglycans. These approaches will hopefully provide valuable information to assist our understanding of muscle connective tissue and meat quality.

# REVIEW OF LITERATURE

## I. MUSCLE CONNECTIVE TISSUE

Connective tissue provides a framework to support organs and tissues in the animal body. Muscle connective tissue is composed of the epimysium, perimysium, endomysium and associated tendons. The epimysium, a heavy sheath of connective tissue, covers the muscle as a whole. The perimysium, which originates in the inner surface of the epimysium, separates the muscle fibers (muscle cells) into bundles or fasciculi. The delicate connective tissue surrounding each muscle fiber is called the endomysium. The connective tissue of the epimysium, the perimysium and the endomysium is continuous with the connective tissue of the tendon by which muscle fibers are attached to bones or other structures (Ham and Lesson, 1961).

Light microscopic studies have grouped connective tissue components into three classes:

(1) cells, such as fibroblasts, adipocytes and mast cells;

(2) fibers, such as collagen, elastin and reticulin; and

(3) the ground substance (or matrix) of which the major components are mucopolysaccharides (MPS).

The MPS are now more frequently referred to as the glycosaminoglycans (GAG)[1] (Mathews, 1968). The cell and fiber components are embedded

---

1. In general, the nomenclature followed in this thesis is according to Balazs and Jeanloz (1965) and Balazs (1970).

in the ground substance of the connective tissue.

Literature values for the concentrations of collagen, elastin and GAG in muscles of domestic animals are shown in Table 1.

## II. CHEMISTRY AND PHYSIOLOGY OF CONNECTIVE TISSUE COMPONENTS

### 1. Collagen

Collagen, which is the major protein component of connective tissue, is also the most abundant protein found in mammals, accounting for about 25% of their total protein (Bailey, 1968). The mechanical stability of an animal's body depends almost entirely upon collagen. During muscle contraction, collagen is well adapted to the role of transmitting tensile force (Bailey, 1968).

Collagen appears as bundles of individual nonbranching fibrils aligned in parallel (Gross, 1961). The collagen molecule is made up of three polypeptide chains (α- chains). Four different molecular types are known to exist in vertebrate tissues:
Type I ($[\alpha 1(I)]_2\alpha 2$), Type II ($[\alpha 1(II)]_3$), Type III ($[\alpha 1(III)_3]$) and Type IV ($[\alpha 1(IV)]_3$) (Miller and Matukas, 1974). Each differs with respect to chain composition and primary structure of component α-chains. Bailey (personal communication) found that intramuscular collagen is a mixture of Type I and Type III in contrast to tendon collagen which is almost pure Type I. The type of collagen found in epimysial tissue, which has not been reported so far, may be similar to that of tendon collagen. Each chain of the collagen

Table 1. Concentration of Muscle Connective Tissue Components

| Author | Animal | Muscle | Age | Collagen (mg/g)[1] | Elastin (mg/g)[1] | GAG Hexosamine (µg/g)[1] |
|---|---|---|---|---|---|---|
| | | (Intramuscular tissue) | | | | |
| Wilson et al. (1954) | Cattle | LD[2] | Veal | 29.6 | 10.3 | |
| | | | Steer | 18.9 | 5.2 | |
| | | | Aged Cow | 18.4 | 4.9 | |
| Cross et al.[3] (1973) | Cattle | SM | | 24.99 ± 8.99[4] | 23.72 ± 10.94[4] | |
| | | ST | | 34.26 ± 11.74 | 34.85 ± 12.80 | |
| | | BF | 0.8 to | 41.27 ± 13.33 | 21.95 ± 12.37 | |
| | | RF | 14.0 years | 39.81 ± 19.82 | 21.13 ± 7.66 | |
| | | LD | | 28.56 ± 11.36 | 13.88 ± 7.21 | |
| Gilbreath et al. (1971) | Cattle | TB | Calf | | | 143.5 |
| | | | Heifer | | | 96.6 |
| | | | Old Cow | | | 111.9 |
| Fox (1968) | Cattle | LD, SM, and TB | 1 to 5 years | | | 273.5[5] |
| Wipf et al. (1970) | Pig | Ham & Loin | | | | 418.5[6] |
| | | (Epimysial tissue) | | | | |
| Field et al. (1970) | Cattle | LD | Old Cow | 707.14 ± 47.31[7] | | |
| | | BF | Old Cow | 638.25 ± 48.41 | | |

Table 1. (continued)

| Author | Animal | Muscle | Age | Collagen (mg/g)[1] | Elastin (mg/g)[1] | GAG Hexosamine (μg/g)[1] |
|---|---|---|---|---|---|---|
| Cormier et al. (1971) | Cattle | SM | Veal | | | 700[8] |
| | | | Steer | | | 534 |
| | | | Heifer | | | 498 |
| | | | Old Cow | | | 408 |

[1]Lyophilized-defatted basis unless specified.

[2]See list of abbreviations.

[3]Found to be intramuscular collagen and elastin from the title of the reference, although whether epimysium is free or not is uncertain.

[4]Standard deviation.

[5]Mean GAG concentration expressed as glucosamine HCl (325.9 μg/g) from LD, SM and TB intramuscular tissue by this author was converted to glucosamine basis.

[6]Pooled value of mean hexosamine concentrations from DS and residual GAG mixture in normal muscle.

[7]Lyophilized basis.

[8]μg hexosamine per g sample, which was calculated from the hexosamine concentration of the lyophilized GAG reported by these authors.

molecule is composed of about one thousand amino acid residues. The three α-chains form a triple stranded helix, in which they are held together in part by hydrogen bonds between the oxygen atoms and the nitrogen atoms of peptide linkages in adjacent chains (Gross, 1961; Bodwell and McClain, 1970; Bornstein, 1974).

Many of the unique chemical characteristics of collagen are due to its relatively high content of glycine, proline, hydroxyproline and alanine (Bodwell and McClain, 1970). Hydroxyproline and hydroxylysine are amino acids unique to collagen and elastin (Bailey, 1968).

The collagen molecule is stabilized by means of intra and intermolecular crosslinks. The biochemical mechanism of crosslinkage formation in collagen has not been completely elucidated. However a number of the biochemical steps have been discussed in a recent review by Tanzer (1973). Specific lysine and hydroxylysine residues are oxidized by the enzymatic action of lysyl oxidase and converted to aldehydes. The aldehydes, α-amino-adipic acid δ-semialdehyde and δ-hydroxy α-aminoadipic acid δ-semialdehyde, react with each other within the collagen molecule to form a stable aldol condensation product, which is an intramolecular crosslinkage. The aldehydes may also react with lysine, hydroxylysine or histidine or another aldehyde in the adjacent collagen molecule to form a reduced Schiff base product or an intermolecular crosslinkage.

Collagen fibers are converted in large part to soluble gelatin by boiling in water or in acid (Cassens, 1970). Collagen denatured in

this manner is generally separated into α, β and γ components depending upon the extent of cross-linkage between each α-chain. If there is no covalent cross-linkage between the α-chains, one muscle collagen molecule yields three α-chains, two α1 and one α2. If there are intramolecular cross-linkages between two α-chains, or among three α-chains, these two or three chains do not separate and remain as a unit in solution. A two chain unit is called a β-component, while a three chain unit is called a γ-component (Laakkonen, 1973; Veis, 1964).

Millar and Matukas (1974) have recently reviewed collagen biosynthesis. Hydroxylation of proline and lysine occurs on nascent α-chains. Procollagen molecules formed through the steps of chain assembly, helix formation and glycosylation, are released into the extracellular space by exocytosis and are converted to collagen molecules by a proteolytic enzyme, procollagen peptidase.

2. Elastin

Unlike collagen, elastin is an extremely stable yellow fibrous protein. Elastin fibers freely branch and anastomose. The elastin molecule is rich in hydrophobic amino acids and therefore readily forms a globular structure in physiological solutions. Elastic fibers are scarce in muscle, with a few exceptions such as the bovine semi-membranosus (SM) which has been reported to contain approximately 35 mg elastin per g lyophilized-defatted tissue (Cross et al., 1973). Elastin contains from 1 to 2% hydroxyproline. A considerable proportion of the lysine found in elastin is covalently bound in two amino acids unique to elastin, desmosine and isodesmosine. The concentration of these two amino acids increases with increasing age

of the animal and imparts chemical stability to the elastin molecules.

3. Reticulin

Reticulin is the least understood of the connective tissue fibrous proteins (Bodwell and McClain, 1970). Reticulin fibers are fine, highly branched, and intimately associated with the endomysium (Cassens, 1970). The nature of reticulin and whether or not it is chemically related to collagen and yields gelatin upon hydrolysis has long remained a subject of controversy (Bodwell and McClain, 1970). Robb-Smith (1958) classified the reticulins into two groups: collagenous and noncollagenous. Histochemically, the reticulin fiber is stained black by ammoniacal silver solution, and is therefore called the argyrophilic fiber (Bodwell and McClain, 1970). Quantitative analysis of muscular reticulin has not yet been reported in the literature.

4. Glycosaminoglycans

(i) Type and Chemistry of GAG

The connective tissues of higher animals are known to contain the following eight well characterized GAG:

(1) Hyaluronic acid (HA)

(2) Chondroitin (Ch)

(3) Chondroitin 4-sulfate or Chondroitin sulfate A (Ch4-S)

(4) Chondroitin 6-sulfate or Chondroitin sulfate C (Ch6-S)

(5) Dermatan sulfate (DS)

(6) Heparan sulfate or Heparitin sulfate (HS)

(7) Heparin (HP)

(8) Keratan sulfate (KS)

Table 2 presents a detailed description of the components of these GAG. Glycosaminoglycans are relatively long polysaccharide chains containing repeating disaccharide units made up of one mole of hexosamine and one mole of uronic acid (or galactose in the case of KS).

There are two isomeric hexosamines found in GAG, glucosamine and galactosamine. Glucosamine containing GAG (HA, HS, HP and KS) are also called glucosaminoglycans, while galactosamine containing GAG (Ch, Ch4-S, Ch6-S and DS) are called galactosaminoglycans (Balazs and Jeanloz, 1965). Except for HA and Ch, the hexosamine is either O or N sulfated in the 4th or 6th position. There are two isomeric uronic acids[1] found in GAG, glucuronic acid and iduronic acid. It is generally agreed that only glucuronic acid is present in HA, Ch4-S and Ch6-S , while both uronic acids are present in DS, HS and HP.

Connective tissue GAG are covalently bound to protein forming complexes known as proteoglycans. The existence of an HA proteoglycan has not been established with certainty (Dorfman, 1974). The polysaccharide chain is bound to protein by means of a galactosyl-galactosylxylosylserine linkage in the case of Ch4-S, Ch6-S, DS, HP

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1. Galacturonic acid has been found in polysaccharide preparations obtained from human brain (Stary et al., 1964), snail mucus mucin (Masamune and Yoshizawa, 1956) and a wide variety of plants (Seno et al., 1972).

Table 2. GAG Composition[1]

| GAG[2] | Components of repeating disaccharide units | N-Acetyl groups | O-Sulfate groups | N-Sulfate groups |
|---|---|---|---|---|
| HA | D-GlcN and D-GlcUA | + | - | - |
| Ch | D-GalN and D-GlcUA | + | - | - |
| Ch4-S | D-GalN and D-GlcUA | + | + | - |
| Ch6-S | D-GalN and D-GlcUA | + | + | - |
| DS | D-GalN and { D-IdUA[4] / D-GlcUA | + | + | - |
| HS | D-GlcN and { D-GlcUA[4] / D-IdUA | + | + | + |
| HP | D-GlcN and { D-GlcUA[4] / D-IdUA | +[3] | + | + |
| KS | D-GlcN Gal | + | + | - |

[1] From Rodén et al. (1972).

[2] See list of abbreviations.

[3] A minor proportion of the amino groups are acetylated, while most are sulfated.

[4] Uronic acid may be either D-IdUA or-GlcUA, with first listed most common.

and HS, where there is an O-glycosidic linkage between xylose and the hydroxyl group of serine in peptide linkage (Stoolmiller and Dorfman, 1969; Rodén et al., 1972). Major anatomical locations of GAG are shown in Table 3.

(ii) Additional GAG (Oversulfated GAG)

Recently small amounts of oversulfated forms of ChS[1] and DS have been reported in fish and mammalian tissues. These forms contain more than one sulfate group per disaccharide unit (Yoshizawa, 1972). The presence of these GAG in mammals has been demonstrated in porcine skin (Suzuki et al., 1968; Malmström and Fransson, 1971), bovine lung (Suzuki et al., 1968), human aorta (Harada et al., 1969), bovine brain (Saigo and Egami, 1970), human urine (Varadi and Griffiths, 1971; Murata et al., 1971) and human cartilage (Iwata, 1969, cited by Yamagata, 1969).

(iii) Heterogeneity of GAG

During polysaccharide biosynthesis, there is no direct transcription of the carbohydrate sequences. This suggests a high probability that heterogeneous polysaccharide macromolecules will be synthesized (Meyer, 1970). It has to be noted here that the repeating disaccharide units of the same type of GAG are not necessarily identical. For example, Fransson and Rodén (1967) and Fransson and Havsmark (1970) extensively studied the molecular structure of DS.

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1. ChS include both Ch4-S and Ch6-S.

Table 3. Major Anatomical Locations of Individual GAG[1]

| GAG | Anatomical Locations |
|---|---|
| HA | Vitreous humor, umbilical cord, brain, cartilage, skin, blood vessels, vitreous and synovial fluids, serum, chicken comb, tendon, epimysial tissue[2], etc. |
| Ch | Bovine cornea. |
| Ch4-S | Cartilage, bone, tendon, cornea, sclera, teeth, aorta, serum, brain, nucleus pulposus, urine, etc. |
| Ch6-S | Cartilage, bone, tendon, skin, aorta, teeth, sclera, heart-valve, nucleus pulposus, urine, etc. |
| DS | Skin, tendon, heart-valve, aorta, lung, epimysial tissue[2], urine, etc. |
| HS | Lung, liver, aorta, urine, etc. |
| HP | Liver, lung, kidney, muscle, blood vessels, heart, rat skin, etc. |
| KS | Nucleus pulposus, cartilage, aorta, annulus, etc. |

[1] From Brimacombe and Webber (1964) and Seno (1968).

[2] Reported by Cormier et al. (1971).

These authors reported three different types of repeating disaccharide units depending upon species and tissues studied. Evidence of heterogeneity in other GAG molecules has also been reported by numerous researchers, e.g., Lindahl (1966) for HP, Hovingh and Linker (1974) for HS, Seno et al. (1965) for KS. Possibly HA is an exception (Meyer, 1970), as heterogeneity in this abundant GAG has not been reported to this time.

(iv) Muscular GAG

There is very little information available concerning the amount and types of GAG found in muscle. In 1966, McIntosh reported ChS to be the only GAG present in bovine intramuscular tissue. This author expressed some uncertainty as to whether ChS accounts for the total intramuscular GAG since only 10% of the total intramuscular hexosamine was extracted. Fox (1968), Wipf (1969) and Wipf et al. (1970) reported the presence of HA, Ch, DS and HP in addition to ChS in bovine and porcine intramuscular tissues. These authors, however, did not thoroughly characterize these GAG. Gilbreath et al. (1971) reported the presence of HA and ChS (or DS) in bovine intramuscular tissue. Cormier et al. (1971) determined the presence of HA, DS and ChS in bovine epimysial tissue by electrophoretic techniques.

Quantitative analysis suggests that considerable variation exists in bovine total intramuscular GAG hexosamine concentrations. For example, Gilbreath et al. (1971) reported the total triceps brachii (TB) intramuscular GAG hexosamine concentration in a 15 month old heifer to be 96.6 µg per g on a lyophilized-defatted basis, while Fox

(1968) reported average GAG hexosamine concentrations in bovine longissimus dorsi (LD), SM and TB intramuscular tissues from animals 1 to 5 years old to be 273.5 µg per g on a lyophilized-defatted basis (Table 1). Thus very little is known about muscular GAG.

(v) Biosynthesis of GAG

Glycosaminoglycans are mainly produced in fibroblasts and mast cells (Dorfman, 1970, 1974; Riley, 1959). Uridinediphosphate (UDP)-acetylglucosamine and UDP-acetylgalactosamine are the precursors for the hexosamine constituents, while UDP-glucuronic acid and UDP-iduronic acid are the precursors for the uronic acid constituents. The order of synthesis of the ChS components has been suggested to be: protein, the galactosylgalactosylxylosyl bridge structure followed by addition of the repeating disaccharides units (Suzuki, 1968). Sulfate groups are added to the growing polysaccharide chain from 3'-phospho-adenosine 5-phosphosulfate (McGilvery, 1975). These steps likely occur in the Golgi apparatus (Revel, 1970).

(vi) Physiological Functions of GAG

Physiologically all substances entering or leaving cells must pass through the surrounding ground substance of the extracellular space in which the GAG are a major component. Therefore the state and composition of the GAG may exert a profound influence on the biological function of cells and tissues (Brimacombe and Webber, 1964). Ogston (1970) suggested that since GAG are large, flexible and chain-like molecules, they have filtration, exclusion and lubrication effects in the animal body. Mathews (1967) has suggested that GAG contribute

to physiological functions such as water balance, ionic homeostasis, calcification, blood coagulation, collagen fibrillogenesis, mechanical support, etc. Glycosaminoglycans may play a significant role as ion exchangers in tissues, acting either as a reservoir for specific ions or by controlling ion movement across membranes. The polyanionic groups of the polysaccharides are always accompanied by cations ($Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$ etc.), which are intimately associated with normal cell function. Thus the large number of anionic groups gives the GAG molecule the property of a modified ion exchanger, where some of the cations may be exchanged with other cations under specific conditions (Mathews, 1967; Fox, 1968).

Hyaluronic acid, a very diffuse molecule having a high viscosity and water binding capacity, occupies a volume many times as large as its actual size. Schubert (1964) estimated that HA occupies a space 25,000 times as large as an equal weight of collagen. Meyer (1947) has suggested that HA functions by binding water in the interstitial spaces and by holding cells together in a jelly-like matrix. It has also been suggested that the viscosity of HA imparts to joint fluids their lubricating and shock-absorption properties (Ogston and Stainer, 1953). Hyaluronic acid in connective tissue interfibrillar regions may act as a protective agent against bacterial attack (Brimacombe and Webber, 1964).

Several investigators have postulated a role for ChS in the calcification of bone (Rubin and Howard, 1950; Sobel, 1955; Lacroix, 1956).

It is well known that HP has blood anticoagulant and lipoprotein lipase activating properties (Mathews, 1968). These two properties have also been observed for HS (Grossman and Cifonelli, 1962) and anti-coagulant activity of DS (Grossman and Dorfman, 1957).

Information concerning the physiological role of GAG in muscle is extremely limited. Muscle GAG may have important roles in water balance, cation transport, translation of hormonal messages, cell division, mechanical support and other physiological functions.

5. Glycoproteins and Sialic Acid.

Glycoproteins are conjugated proteins containing one or more heterosaccharides, usually branched, lacking a serially repeating unit and bound covalently to the polypeptide chain (Gottschalk, 1972). Sialic acid (N-acetylneuraminic acid) is found as a component of the heterosaccharide chain. Sialic acid containing glycoprotein is found widely distributed throughout the animal kingdom, for example in mucus secretions, blood group substances, milk and egg proteins, hormones and enzymes, as well as in connective tissue (Spiro, 1970).

More information about sialic acid and glycoproteins is available elsewhere (Gottschalk, 1960; Gottschalk, 1972), although very little is known about their role in muscle.

## III. MUSCLE CONNECTIVE TISSUE CHANGES WITH AGE

### 1. Collagen

From the quantitative aspect, there are numerous reports on age associated collagen analyses of intramuscular tissues, although there

is little information available concerning the quantitative aspects of epimysial or fetal muscular tissues.

Wilson et al. (1954) have reported the intramuscular collagen concentration of the LD to be higher in veal than in beef from aged cows on a lyophilized-defatted basis. Similarly, Vognarová et al. (1968) observed a higher collagen concentration in veal (approximately 3 weeks of age) than in beef (approximately 2 years of age) in several different muscle cuts such as fillet, round, loin, flank, ribs, neck and shank. Cross et al. (1973) studied four major intramuscular tissues from three bovine age groups (average ages: 483, 1376 and 4087 days), and reported no significant difference in collagen concentration among these age groups on a lyophilized-defatted basis. Similarly, Hill (1966) found no significant difference in the collagen concentration of bovine sternomandibularis muscle from animals ranging in age from 4 months to 4.5 years on a lyophilized basis.

Cormier et al. (1971) studied bovine epimysial collagen from SM muscles and reported that collagen concentration, on a lyophilized-defatted basis, is significantly higher in new born animals (2 to 3 days) and steers (11.5 months) than in heifers (14.5 months) and cows (9 to 10.5 years). These authors suggested that the higher concentration of collagen in veal is in agreement with the veal intramuscular collagen concentration data of Goll et al. (1963) and Carmichael and Lawrie (1967). However, in other tissues such as skin, blood vessels and joints, which are almost entirely composed of connective tissue as is the epimysial tissue, collagen concentration generally increases with age (Bailey,

1968). Kondo et al. (1971) studied chicken skin from three age groups (newly hatched, 4 weeks and 23 months) and reported that hydroxyproline concentrations in lyophilized-defatted skin were found to be 3.36, 5.40 and 10.33% respectively. Although information concerning epimysial collagen is limited, the above age related observations by Cormier et al. (1971) are likely in error. This point will be discussed later in this thesis.

From the qualitative aspect, the thickness of collagenous tissue in muscle, as determined by histochemical techniques, increases with age (Carpenter et al., 1963). Furthermore, it is well established that with increasing age, collagen steadily increases in stability as reflected by characteristic phenomena such as a decrease in its solubility in salt and acid solutions (Houck et al., 1961; McClain et al., 1965; Carmichael and Lawrie, 1967; Kruggel and Field, 1974) and alkali solutions (Kao and McGavack, 1959; Cormier et al., 1971), susceptibility to enzymatic degradation (Goll et al., 1964a), heat lability (Hill, 1966; Cross et al., 1973), and swelling capacity (Banfield, 1956; Bakerman, 1962) as well as an increase in its tension generated during isotonic thermal contraction (Verzár, 1964). In addition, the β and γ components, more stable forms of denatured collagen, are present in greater amounts in collagen from older animals than in collagen from younger animals (Veis, 1970).

To account for these increases of collagen stability with increasing age, it was proposed by Verzár (1964) that the extent of collagen crosslinkage increases as an animal grows older. More recently, Davis et al. (1975) studying bone and dentine collagen

maturation in vitro, have reported that the reducible crosslinks are gradually transformed into more stable, nonreducible crosslinks during maturation. According to these authors, collagen maturation involves nucleophilic addition of lysine and/or hydroxylysine residues to the electrophilic double bond of the reducible crosslinks, yielding more stable derivatives. Shimokomaki et al. (1972) have also demonstrated a decreased number of reducible crosslinks in bovine intramuscular collagen with increasing age.

2. Elastin

Elastin in muscle has been less extensively studied than collagen, perhaps because it forms a lesser proportion of total connective tissue, and because of the inherent difficulty of isolating this relatively stable protein (Cross et al., 1973).

From a quantitative point of view, Wilson et al. (1954) have reported a higher elastin concentration in the LD muscle from veal than from aged cows on a lyophilized-defatted basis. However, more recently the elastin concentration in several muscles, including the LD muscle, has been shown to be relatively constant between veal (approximately 3 weeks of age) and beef (approximately 2 years of age) (Vognarová et al., 1968) and among three groups of cattle ranging from 483 to 4087 days of age (Cross et al., 1973).

The number of elastin crosslinkages increases with age through the formation of desmosine and isodesmosine which are the aldol or Schiff base condensation products derived from four lysine residues, resulting in a decrease of the ε-amino group nitrogen content of elastin

(Piez, 1968; Gallop et al., 1972; Francis et al., 1973).

3. Reticulin

There is little information available about reticulin. Using histochemical techniques, Gross (1950) observed a decrease in reticulin, and an increase in the collagen staining reaction of rat skin with increasing age.

4. Glycosaminoglycans

Only a limited number of determinations of muscular GAG concentrations have been reported.

Gilbreath et al. (1971), studying bovine TB intramuscular GAG from three animals (3 month old calf, 15 month old heifer and 9 year old cow), reported GAG concentrations of 356.5 ± 18.3, 239.3 ± 13.6 and 256.5 ± 14.1 μg per g respectively in lyophilized-defatted preparations. Cormier et al. (1971) studied bovine SM epimysial tissue from newborn to 10.5 year old animals and reported that the amount of isolated GAG decreased with increasing animal age with a correlation of -0.63 ($P < 0.05$).

However, relatively little is known about muscle GAG at this time (Laakkonen, 1973). This thesis deals in considerable detail with the GAG of muscle; therefore it is worthwhile to look at the effect of age on GAG in other tissues which have been more extensively studied. A number of changes in the ground substance of various tissues from a number of species have been shown to be associated with increasing age. These changes include a decrease of ground substance

(Bailey, 1968), ground substance water (Dennis, 1959), GAG concentration (Clausen, 1963; Torii et al., 1965; Kondo et al., 1971), hexosamine concentration (Houck et al., 1961), hexosamine:hydroxyproline ratio (Clausen, 1962a) and hexuronic acid:hydroxyproline ratio (Clausen, 1962b).

Kaplan and Meyer (1959) reported a linear decrease in the concentration of ChS in human cartilage with age. The concentration of KS was found to be negligible in the very young human,to reach a plateau at 20-30 years of age and thereafter to remain constant, accounting for approximately 50% of the total GAG. When the ratio of KS to total GAG was plotted against age, a linear increase was obtained from the newborn to individuals 74 years of age.

Loewi and Meyer (1958) studied pig skin and observed that DS, HA and Ch6-S changed as a percentage of the total skin GAG from about 5 — 12%, 78% and 20%,to 64%, 30% and 0.1% respectively from embryos to adults. Rat skin HP concentration has been observed to diminish markedly (75%) after 50 days of age, then remain relatively constant (Schiller and Dorfman, 1960). In the human aorta of individuals ranging in age from 21 to 72 years, HA and Ch6-S concentrations decrease, while DS and HS concentrations increase as a percentage of the total GAG (Kaplan and Meyer, 1960). Recent studies of human urinary excretion of GAG have shown that the total GAG excreted decreases, while the proportion of low sulfated chondroitin sulfate (Ohkawa et al., 1972) and hyaluronidase resistant GAG (HS, DS and KS) increase with advancing age (Allalouf and Ber, 1970).

5. Glycoproteins and Sialic Acid

There is very little information available in the literature concerning glycoprotein and sialic acid concentration changes with age in either muscle or other tissues.

It has been observed that the concentration of non-amino sugar constituents in neutral glycoprotein of rat skin and ox aorta decreases (Hakomori, 1962), and that the concentration of sialic acid in the human cornea and lens increases with age (Haddad, 1962).

## IV. SEX DIFFERENCES IN MUSCLE CONNECTIVE TISSUE

Bovine muscle connective tissue from animals of different sexes has been studied by Field et al. (1969). Bulls 20 months of age were found to have a thicker epimysial tissue surrounding the LD than steers of the same age. The hydroxyproline concentration determined in the lyophilized LD muscle was found to be lower in the steers, indicating a lower collagen concentration in steers than in the bulls. In histochemical analysis of collagen and elastin in these same muscles, these authors recorded a lower subjective score for these proteins in steers than in bulls.

Kao and McGavack (1959) reported some sex differences in rat tissues. The insoluble collagen concentration in abdominal muscle was found to be higher in females than in males ranging from 3 weeks to 8 months of age. The collagen concentration of tendons was found to be greater in male than in female rats at ages ranging from 5 weeks to 2 years. The elastin concentration of the aorta was found to be higher in the male than in the female at 8 months of age.

Cembrano et al. (1960) studied chicken aorta and found a significantly higher amount of elastin and collagen in males than in females.

Herrick and Brown (1952) reported that there was a decrease in the tensile strength and the per cent collagen nitrogen in the muscle and skin after old cocks were caponized or treated with diethylstilbestrol, and that capons treated with testosterone propionate developed an increased tensile strength and collagen level in these same tissues.

Gilbreath et al. (1971) reported a higher concentration of intramuscular HA in a 9-year-old cow than in a 15-month-old heifer and suggested that this result may be due to hormonal changes associated with lactation. Cormier et al. (1971) suggested that the reduced level of estrogens in aged cows and the absence of estrogen and testosterone in steers was responsible for the low epimysial HA concentration in these two groups of animals. On the other hand, Fox (1968) reported no significant sex differences in muscle hexosamine content of the various GAG fractions.

## V. MUSCLE CONNECTIVE TISSUE AND MEAT QUALITY

There are numerous factors contributing to meat quality, such as tenderness, juiciness, flavor, color and texture (Lawrie, 1966). Of these, tenderness is one of the most important palatability factors in the acceptance of meats (Bratzler, 1970).

For convenience, factors influencing meat tenderness can be divided into two general categories. One is influenced by the animal's

own traits, including age, sex and breed, and the other is influenced by the pre-or post-slaughter handling which affects post-mortem changes of muscle (Nakamura, 1973).

In the first category, connective tissue, particularly collagen, is the main contributing factor to meat toughness (Bailey, 1972; Shimokomaki et al., 1972; Nakamura, 1973; Lawrie, 1968).

The amount of connective tissue differs among muscles from different anatomical locations within an animal (Briskey and Kauffman, 1970). Meat which has more or thicker connective tissue (e.g., shank) is generally considered to be tougher than meat which has less or thinner connective tissue (e.g., tenderloin (Briskey and Kauffman, 1970). Cover et al. (1962) and Ritchey et al. (1963) cooked beef to 61 C (rare) and to 80 C (well-done) and determined meat tenderness by scoring. Scores for tenderness were lower (tougher) and collagen content was higher in biceps femoris (BF) muscle than in LD muscle. Similarly, chicken wing muscle, which contains less collagen than thigh muscle is more tender (Nakamura, 1973).

Meat from the same anatomical location is normally more tender in younger than in older animals. However in comparing these groups of animals, there is not always a negative correlation between collagen content and meat tenderness (Goll et al., 1963; Herring et al., 1967). For example, veal which has a higher concentration of collagen than beef (Wilson et al., 1954; Vognarová et al., 1968) is well known to be much more tender (Cormier et al., 1971), suggesting a positive correlation between collagen content and meat tenderness. With regard to this age

associated meat toughness paradox, Bailey (1972) suggested that not only total collagen but also the quality of the collagen affects meat toughness, and that the ratio of thermally stable bonds to labile Schiff bases is an important factor. This concept is consistent with the reports of Field et al. (1969) and Cross et al., (1973) who observed a significant decrease of labile collagen (i.e., an increase of stable collagen crosslinks) with a concomitant increase of meat toughness.

Concerning the second category of meat tenderness, the post-mortem tenderization of muscular connective tissue, early researchers found no change in the alkali-insoluble portion of collagen (Wierbicki et al., 1954; Khan and Van der Berg, 1964; de Fremery and Streeter, 1969) during post-mortem aging. These studies of the gross solubility of collagen suggested that collagen, a more stable protein than myofibrillar protein, does not undergo a significant change during post-mortem aging. McClain et al., (1965) reported a decrease in salt-soluble collagen and a concurrent increase in acid-soluble collagen in steer, calf and lamb fore-shank muscle aged seven days post-mortem. More recently, McClain et al. (1970) reported the amount of intramuscular connective tissue (IMCT) extracted from bovine muscles frozen in dry ice at 72 h post-mortem was 50% lower than that at 0 h. Similarly, a 34% decrease was found for porcine muscle. However these authors did not provide information concerning the purity or the yield of the IMCT extracted.

Structural studies made on the collagen molecule during post-mortem aging by acrylamide-gel electrophoresis have indicated that some changes

occur in the sub-unit composition of intramuscular guanidine hydrochloride-soluble collagen. For example, Pfeiffer et al. (1972) found an increase in the α-component and a decrease in the β and γ components during post-mortem aging. These authors also found that muscle stretching during this period increased the relative amount of the α-component.

The literature contains few reports relating GAG and meat quality. Cormier et al. (1971) found no significant correlation between shear value and the GAG fractions obtained chromatographically. Fox (1968) studied GAG fractions and meat tenderness in bovine intramuscular tissues, and also found no significant correlation between these two measurements. On the other hand, Wipf (1969) and Wipf et al. (1970), studying porcine intramuscular tissues, reported a significant positive correlation ($P < 0.01$) between shear value and DS concentration. These authors also reported that pale, soft, exudative pork contains a significantly ($P < 0.05$) higher DS concentration than normal pork. However it has to be noted here that these authors tentatively identified proteolytic enzyme undigested muscle residue (a very crude preparation) to be DS without performing the appropriate characterizations. Glycosaminoglycans have been also studied with regard to post-mortem aging in bovine intramuscular tissue. Fox (1968) reported that the intramuscular hexosamine concentration obtained in four GAG fractions (0.4, 1.25, 1.5 and 2.0 M NaCl) did not differ significantly during seven days' post-mortem aging.

The study reported in this thesis was carried out to elucidate age and sex associated changes in bovine muscular connective tissue composition. A major emphasis is placed on the quantitative and qualitative analysis of GAG, an area poorly understood as applied to meat producing animals.

## MATERIALS AND METHODS

### I. EXPERIMENTAL ANIMALS

The experimental animals used in this study were obtained from the University of Alberta Ranch or from selected animals being processed by Gainers' Ltd., Edmonton. Details of the age, sex, breed and source of all experimental animals are presented in Table 4. Trial I was comprised of fetuses and animals of known history, while Trial II was comprised of animals of unknown history.

### II. SAMPLING AND PREPARATION OF MUSCLE

The triceps brachii muscle (TB muscle), which has been used for GAG research by other authors (Fox, 1968; Gilbreath et al., 1971), was selected for this project.

All tissue samples were collected at a slaughter house (Gainers' Ltd.) while the experimental animals were being processed by routine procedures. Three 5 x 5 x 10 mm tissue samples, for histochemical analyses, were removed from the central surface of the left TB muscle of each animal within 60 min after slaughter. These samples were retained for tissue fixation in a 20 ml glass vial containing 10 % formalin solution (formalin, 10% $CaCl_2$ and distilled water, 1:1:8) (Baker, 1958).

The whole muscle was removed from the carcass 48 h after slaughter. Individual fetal TB muscles were considered to be too small for some analytical purposes. Muscles from fetuses of similar ages were therefore combined to make two different fetal age groups, 3 to 5 and 6 to 8 months, and each group was divided into two and three replicates, respectively.

Table 4. Experimental Animals

| Group | Trial | Sex | Animal Number | Hot Carcass Weight (lb) | Age (years) | Breed | Source |
|---|---|---|---|---|---|---|---|
| FI | I | - | - | - | Fetus[1] | U[4] | G[7] |
| FII | I | - | - | - | Fetus[2] | U | G[8] |
| I | I | Female | 772-3 | 314 | 1.2 | HeX[5] | K |
| | I | | 777-3 | 260 | 1.2 | HeX | K |
| | I | | 778-3 | 240 | 1.2 | HeX | K |
| IIf | I | Female | 10-2 | 413 | 2.1 | He[6] | K |
| | I | | 46-2 | 380 | 2.0 | He | K |
| | I | | 95-2 | 364 | 2.1 | He | K |
| | I | | 32-2 | 388 | 1.7 | He | K |
| | II | | # 5 | - | 2[3] | U | G |
| | II | | A | - | 2[3] | U | G |
| | II | | B | - | 2[3] | U | G |
| IIm | I | Male | 14-2 | 904 | 1.8 | He | K |
| | I | | 42-2 | 916 | 1.6 | He | K |
| | I | | 40-2 | 934 | 1.6 | He | K |
| | I | | 124-2 | 861 | 1.7 | He | K |
| | II | | 2A | - | 2[3] | U | G |
| | II | | 2B | - | 2[3] | U | G |
| | II | | 2D | - | 2[3] | U | G |
| III | I | Female | 66-7 | 450 | 7.1 | He | K |
| | I | | 16-7 | 563 | 7.2 | He | K |
| | I | | 30-6 | 450 | 7.9 | He | K |
| | I | | 259-5 | 544 | 8.9 | HeX | K |
| | II | | # 3 | - | 7-8[3] | U | G |
| | II | | # 4 | - | 7-8[3] | U | G |

1 Tissue from 20 fetuses 3 to 5 months old was pooled.

2 Tissue from 30 fetuses 6 to 8 months old was pooled.

3 Estimated by a veterinarian.

4 Unknown breeding.

5 Cross bred Hereford.

6 Hereford.

7 Gainers' Ltd., Edmonton.

8 University of Alberta, Kinesila Ranch.

Each TB muscle was trimmed of surface fat and separated into two portions for subsequent analyses; epimysium (epimysial tissue) which was free of lean, and remaining muscle (intramuscular tissue). These two tissues were then individually cut into small pieces, lyophilized and defatted with diethyl ether using a Soxhlet-type apparatus. The presence of peroxides in the diethyl ether was checked daily according to the method of Steere (1967). The average yield of lyophilized-defatted tissue was found to be 22% of the intramuscular tissue and 28% of the epimysial tissue on a fresh tissue weight basis. The lyophilized and defatted tissues were then homogenized in a Waring blender.

The epimysial tissue of fetal muscle was thin and difficult to completely separate from the attached intramuscular tissue. Therefore, only fetal intramuscular tissue was analyzed.

The lyophilized-defatted samples were placed in bottles, tightly sealed, and stored at -30C until analyzed. The moisture content of each sample was checked before each quantitative analysis according to A.O.A.C. (1965) and a correction was made for the small amount of moisture present.

## III. CHEMICALS, REAGENTS AND ENZYMES

All chemicals and enzymes, unless specified otherwise, were purchased from local suppliers and were of analytical grade. All water used was distilled and deionized.

1. Chemicals

Hydroxy-L-proline, D-glucosamine hydrochloride, and D-galactosamine hydrochloride were obtained from Calbiochem, La Jolla, California; p-dimethylaminobenzaldehyde, orcinol, sodium rhodizonate and anthron from Fisher Scientific Co., Fair Lawn, New Jersey; D-glucuronolactone and D-galactose from Eastman Organic Chemicals, Rochester, New York; cetyltrimethyl ammonium bromide and chloramine-T from Eastman Kodak Co., Rochester, New York; cetylpyridinium chloride, bovine serum albumin, 2-thiobarbituric acid, N-acetyl-neuraminic acid, chondroitin sulfate (mixed isomers from whale and shark cartilage, lot 52C-3250), chondroitin sulfate, type A (chondroitin 4-sulfate, from whale cartilage, lot 14C-1730), chondroitin sulfate, type B (dermatan sulfate, from pig skin, lot 102C-2990), chondroitin sulfate, type C (chondroitin 6-sulfate, from shark cartilage, lot 23C-1220), and hyaluronic acid (from human umbilical cord, lot 13C-0520) from Sigma Chemical Co., St. Louis, Missouri; unsaturated disaccharides, ΔDi-0S and ΔDi-6S (lot 1S27) from Miles Laboratories Inc. Canada Ltd., Montreal, Que.

Hyaluronic acid (from human umbilical cord), chondroitin sulfate A (chondroitin 4-sulfate, from rock sturgeon notochord, Acipenser fulvescens), chondroitin sulfate C (chondroitin 6-sulfate, from human umbilical cord), dermatan sulfate (from hog mucosal tissues), heparitin sulfate (heparan sulfate, from beef lung by-products), heparin (from hog mucosal tissues) and keratan sulfate (from bovine corneal tissue) were gifts from Dr. M. B. Mathews, University of Chicago.

Aldehyde free ethanol was prepared according to Dunlup (1906). Carbazole was obtained from Fisher Scientific Co., Fair Lawn, New Jersey, and recrystallized according to the Merck Index (1968). Absolute ethanol for sulfate analysis was prepared by azeotropic means (Young, 1902).

2. Reagents and Buffer Solutions

Chloramine-T and all other reagents used for hydroxyproline analysis were prepared according to Stegemann and Stalder (1967). Ehrlich's reagent was prepared according to Boas (1953). The sulfuric acid reagent for uronic acid analysis was prepared and tested on a water blank prior to sample analysis as outlined by Bitter and Muir (1962). During the water blank test, if a brown or green color was formed, the reagent was rejected for use. Optical densities of the water blank for the reagent used in this study were observed to be 0.012 to 0.020. The orcinol reagent was prepared according to Davidson (1966). The phosphate-citrate buffer (pH 5.0) consisted of 50 ml of 1M citric acid and 330 ml of 0.3M $Na_2HPO_4$ diluted to 1 litre. The cetyltrimethyl ammonium bromide (CTAB) reagent and tris buffer were prepared according to Diferrante (1956) and Saito et al., (1968) respectively. The phosphate buffer solution was prepared according to Hata and Nagai (1972).

3. Enzymes

Crude papain, type II (2.3 Units/mg) and bovine testicular hyaluronidase, type I (360 N.F. Units/mg, lot 33C-2240) were obtained from Sigma Chemical Co., St. Louis, Missouri; pepsin (1:10,000) from Nutritional Biochemicals Co., Cleveland, Ohio; trypsin (1:250) from

Difco Laboratories Inc., Detroit, Michigan; chondroitinase-ABC (5 Units/ampule), chondroitinase-AC (5 Units/ampule), chondro-4-sulfatase (1.6 Units/ampule) and chondro-6-sulfatase (2.5 Units/ampule) (lot KE 3901) from Miles Laboratories Inc. Canada Ltd., Montreal, Que.

## IV. HISTOCHEMICAL ANALYSIS

Collagen, elastin, reticulin and MPS[1] were studied histochemically. Although quantitative observations are difficult by histochemical techniques, the merit of this technique is that tissues can be seen without destroying them. Consequently the site of each component was determined.

### 1. Preparation of Tissues for Microtomy

Preparation of tissues for microtomy was performed according to Drury et al. (1967). Seven μ thick sections (transverse) were cut with an American Optical microtome for subsequent staining.

### 2. Staining Methods

A triple staining procedure for collagen, elastin and reticulin was carried out based on the method of Deethardt and Tuma (1970). The length of the dehydration time with both 90 % and absolute ethanol was reduced from 1 min to 30 sec each after aniline blue staining. In this technique, collagen is stained blue by aniline blue, elastin, red by orcein and reticulin, black by silver.

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1 In the case of the histochemical study, the term MPS instead of GAG was used following the suggestion by Balazs (1970).

Mucopolysaccharides were stained with alcian blue according to Steedman (1950). After staining, sections were mounted with D.P.X. (synthetic resin mountant), a cover glass was attached carefully and the preparation then allowed to dry.

3. Evaluation of Connective Tissue Components

Connective tissue components were evaluated by a scoring method (Ramsbottom et al., 1945). A score ranging from 1 to 7 (1:none, 3: small amount, 5: medium amount, 7: large amount) was assigned to each field observed at a magnification of 970. Three tissue samples from each animal were studied. Each sample consisted of three slides and three sections on each slide were evaluated.

V. ANALYSIS OF TOTAL AND LABILE FRACTION CONNECTIVE TISSUE PROTEINS

Total nitrogen and collagen were determined from each lyophilized-defatted tissue. A labile fraction was then prepared by heating in a salt solution as outlined in section V-2. and subsequently analyzed. All the analyses were performed in duplicate.

1. Quantitative Analysis of Nitrogen

All nitrogen (N) analyses were carried out by the Kjeldahl method (A.O.A.C., 1965).

2. Quantitative Analysis of Collagen

(i) Introduction

Early workers (e.g., Mitchell et al. 1928; Lowry et al. 1941; Irvin and Cover, 1959) estimated muscular collagen concentration as

Kjeldahl nitrogen by isolating connective tissue from muscle by physical or chemical methods followed by autoclaving the connective tissue to separate collagen from elastin by their different heat labilities.

In recent years, the determination of tissue hydroxyproline concentration, which is uniquely high in collagen (13.5%) (Szczesniak and Torgeson, 1965), has become a common means of determining tissue collagen concentrations. In this method, first developed by Newman and Logan (1950), hydroxyproline is liberated from tissue by acid hydrolysis, oxidized with hydrogen peroxide and then reacted with p-dimethylaminobenzaldehyde to form a chromophore. Several modifications of this method (Wierbicki and Deatherage, 1954; Lloyd and Hiner, 1959; Leach, 1960) and another method based on the use of ninhydrin for color development (Troll and Cannan, 1953) have been reported. Recently hydrogen peroxide has been replaced by chloramine-T as the oxidant (Stagemann, 1958; Woessner 1961; Stagemann and Stalder 1967; Kivirikko et al., 1967).

(ii) Hydroxyproline Analysis

A. Selection of a Method

The method of Leach (1960) using hydrogen peroxide as an oxidant was compared to the method of Stagemann and Stalder (1967) using chloramine-T as an oxidant. The latter method, which was found to have greater sensitivity and reproducibility, was used in this study.

B. Tissue Hydrolysis

Tissues are commonly hydrolyzed in 6N HCl at 110 C (McClain et al. 1973). The amount of hydroxyproline liberated from intramuscular and epimysial tissue was determined under these conditions as a function of time (Figure 1). The greatest concentrations of hydroxyproline were found after hydrolyzing both the intramuscular and epimysial tissues for 24 h and the labile fraction for 16 h. These time periods were therefore selected for hydrolysis of these particular experimental preparations.

The destruction of hydroxyproline during hydrolysis was also measured. Five preparations of each of 2 and 4 µg hydroxyproline were heated in 6N HCl at 110 C for 24 h. The mean optical densities of each hydroxyproline preparation were compared with those of their non-hydrolyzed controls. The mean destruction was found to be 3.6%. Carmichael and Lawrie (1967) reported hydroxyproline losses due to hydrolysis (130 C, 3 h in 6N HCl) in the order of 2%.

C. Analytical Procedure

Approximately 0.03, 0.3 and 0.1g lyophilyzed-defatted sample of post natal epimysial, post natal intramuscular and fetal intramuscular tissue, respectively, were placed in 50 ml test tubes with Teflon-lined screw caps and hydrolyzed.

Epimysial tissue hydrolysates were quantitatively transferred into 250 ml volumetric flasks, a small amount of decolorizing charcoal added, and the volume made up with water. Addition of charcoal to a

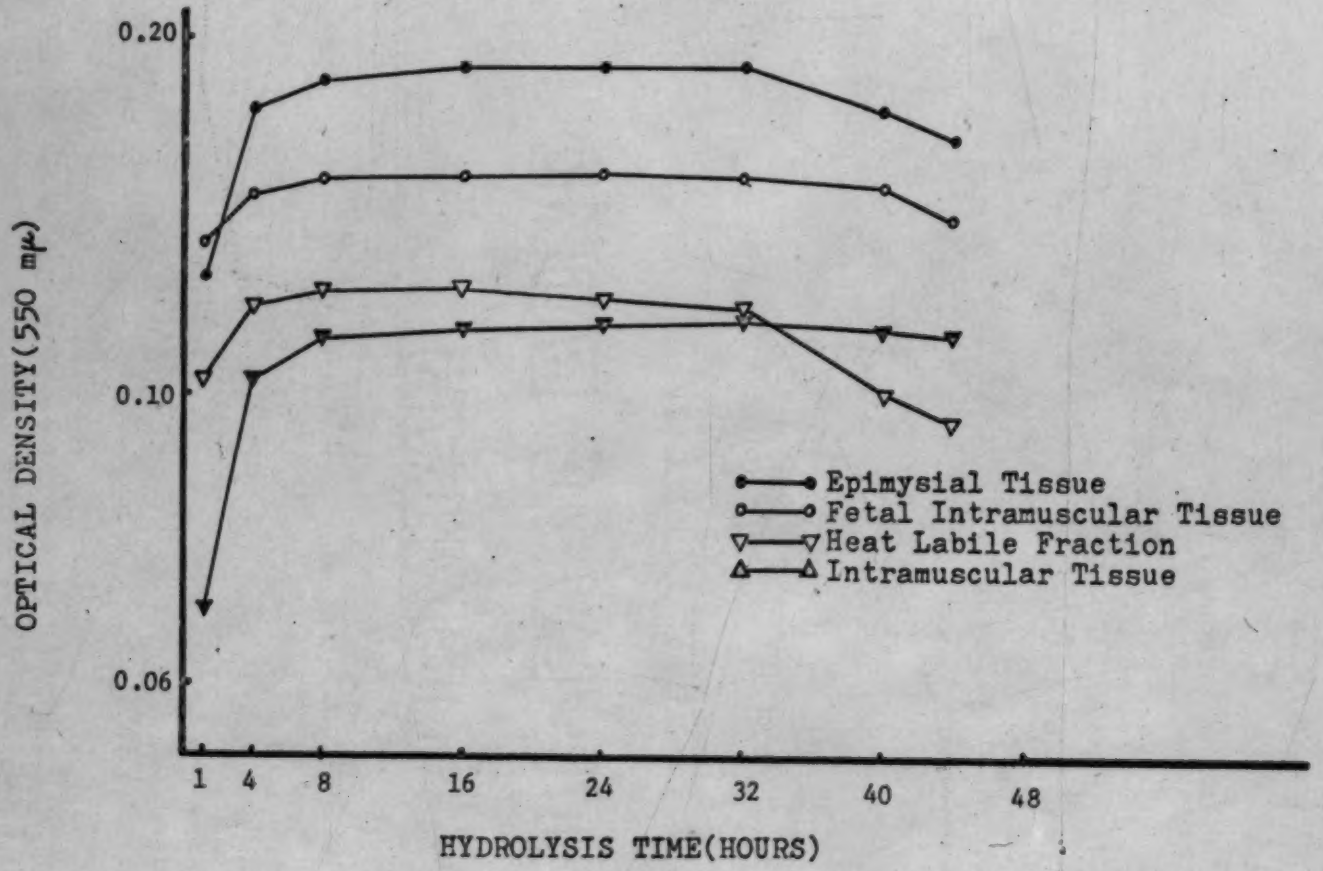



FIGURE 1 Optical density of chromogen formed when each sample was hydrolyzed in 6N HCl at 110 C for various lengths of time. Each value plotted is the mean of three observations.

1000 fold excess of the amount of hydroxyproline does not interfere with this analysis (Stegemann and Stalder, 1967).

In the case of intramuscular tissue, the hydrolysate was filtered through Whatman No. 1 filter paper into a 50 ml-volumetric flask. A small amount of decolorizing charcoal was added and the solution was made up to volume by washing the test tube and filter paper with water.

The diluted hydrolysates were allowed to stand at least 20 min before removal of the decolorizing charcoal by filtration. One to 3 ml of each hydrolysate was then pipetted into a beaker or an evaporation dish and dried in a vacuum oven at 55 C. After cooling to room temperature, 10.0 ml of water was added and the preparation immediately covered with a watch glass. The pH of this preparation was between 6 and 7 as checked by pH paper, which is similar to that reported by Stagemann and Stalder (1967). Aliquots (0.5 to 2.0 ml) of these preparations were subjected to hydroxyproline analysis.

D. Interfering Substances

Stagemann and Stalder (1967) have suggested that one should remove nonspecific chromogens by ion exchange chromatography if experimental tissue hydrolysates contain less than 0.1% hydroxyproline. Although the amount of hydroxyproline in lyophilized — defatted intramuscular tissue hydrolysates was higher than 0.1%, the value of using chromatography was checked by means of 0.5 x 12 cm column containing a Dowex 50 x 8 ($H^+$) 200-400 mesh. No difference was found in the color yields between column treated and non-treated hydrolysates. Thus all muscle tissues were analyzed without column treatment.

It was often found, particularly in intramuscular tissues, that a slight yellow color developed after addition of the chloramine-T reagent. This color, however, disappeared with addition of perchloric acid (same strength as the aldehyde/perchloric acid reagent to be added after chloramine-T reagent in the procedure), which implied that this yellow color is not an interfering chromogen. Stagemann (personal communication), has also observed the development of this yellow color and believes that it has no influence on the analytical results.

(iii) Estimation of Collagen Concentration

The amount of collagen present in each sample was estimated by multiplying the amount of hydroxyproline present by 7.25 (Goll et al., 1963).

3. Preparation and Analysis of the Labile Fraction

(i) Preparation

The labile fraction was prepared from epimysial and intramuscular tissues by the method of Hill (1966) with the exception that 0.15M NaCl was used instead of one-quarter strength Ringer's solution.

Approximately 1.5, 10 and 3 g of lyophilized-defatted epimysial, post natal intramuscular and fetal intramuscular tissue, respectively, were weighed and placed in a 250 ml-plastic centrifuge bottle equipped with a rubber stopper, thermometer, and a vent. Each bottle contained 150 ml 0.15M NaCl and was incubated at 77C for 1 h with occasional gentle shaking. After incubation, the bottles were cooled

to room temperature, and the supernatant removed by centrifugation (5000 x g at room temperature for 10 min). The residue was washed with 100 ml 0.15M NaCl, and recentrifuged. The two supernatants were pooled in a 250 ml-volumetric flask, made to volume with water and used for analyses of hydroxyproline, hexosamine, sialic acid and nitrogen.

(ii) Analyses

For hydroxyproline analysis, 0.5, 1.0 and 0.05 to 0.01 ml aliquots of the epimysial, post natal intramuscular and fetal intramuscular tissue preparations, respectively, were hydrolyzed for 16 hr and evaporated to dryness as outlined previously. A known volume of water (10 to 50, 5 to 10 and 10 to 20 ml for epimysial, post natal intramuscular and fetal intramuscular tissues respectively) was added, and a 1 to 2 ml aliquot was analyzed for hydroxyproline as described previously. Color yields were not influenced by NaCl concentrations as high as 0.2M. Preparations assayed in this study contained less than 0.03M NaCl.

Hexosamine and sialic acid were determined in each labile fraction according to methods presented later in sections VI-2 and VI-7, respectively. The salt was also found not to interfere with sialic acid analysis.

Nitrogen was determined on 50 to 100 ml aliquots of each heat labile fraction. The amount of soluble non-scleroprotein nitrogen (SNSPN) liberated was determined as the difference between total soluble N and soluble collagen N (collagen concentration x 0.1868).

## VI. ANALYSIS OF GAG

### 1. Experimental Approach

Tissues chosen for the study of GAG were limited to those from fetuses and to those from animals of known history from the University Ranch (i.e., Trial I animals). The following two overall types of analyses were carried out. In Analysis I, individual samples from each sex and age group were studied quantitatively by uronic acid analysis and by enzymatic methods. Data obtained were subjected to comparison between age and sex groups.

In Analysis II, samples from each age group were combined, providing larger sample sizes for the estimation of hexosamine recovery and for the detailed characterization of GAG by a number of analytical approaches.

### 2. Quantitative Analysis of Hexosamine

Glycosaminoglycans contain two hexosamines, glucosamine and galactosamine. The literature contains techniques for the quantitative analysis of both hexosamines together as total hexosamine (e.g., Elson and Morgan, 1933) or separately as glucosamine and galactosamine (e.g., Pearson, 1963). In this study, total hexosamine was determined.

#### (i) Quantitative Determination of Total Hexosamine

##### A. Selection of a Method

Colorimetric methods based on the Elson-Morgan (1933) reaction are most commonly used. These methods are based on the condensation of hexosamine with acetylacetone under alkaline conditions, followed by

reaction with Ehrlich's reagent (p-dimethylaminobenzaldehyde) in acidic conditions to produce a chromogen. Numerous modifications of this method (Blix, 1948; Schloss, 1951; Boas, 1953; Gardell, 1953; Belcher et al., 1954; Rondle and Morgan, 1955; Neuhaus and Letzring, 1957; Gatt and Berman, 1966; Maeda et al., 1970) have been reported. Total hexosamine is also determined by using the Morgan-Elson reaction (Suzuki, personal communication) known as the N-acetyl-hexosamine method developed by Morgan and Elson in 1934 and later modified by Reissig et al., 1955, Strominger et al., 1959 and others. Alternative colorimetric methods have been reported (Dische and Borenfreund, 1950; Exley, 1957; Lee and Montgomery, 1961; Tracey, 1952).

Among the reported methods for analysis of total hexosamine, two purportedly reliable methods were carefully studied with reference glucosamine hydrochloride. Gatt and Berman (1966) reported a simple and rapid estimation of total hexosamine with an increased chromogen stability by using propanol instead of ethanol in the Elson-Morgan reaction. This method was studied first. Although these authors reported a maximum color development 5 min after addition of Ehrlich's reagent, which was stable for 1 to 2 h, it was found in this study that an unstable chromogen developed which lost approximately 10% of its optical density within 1 h after addition of Ehrlich's reagent. The method of Boas (1953), which is one of the most common methods based on the Elson-Morgan reaction, was also studied and was found to display an overall high reliability. This method was tested and as shown in Figures 2 and 3, a linear standard curve with reference glucosamine hydrochloride was achieved. The chromogen remained stable 1 h after

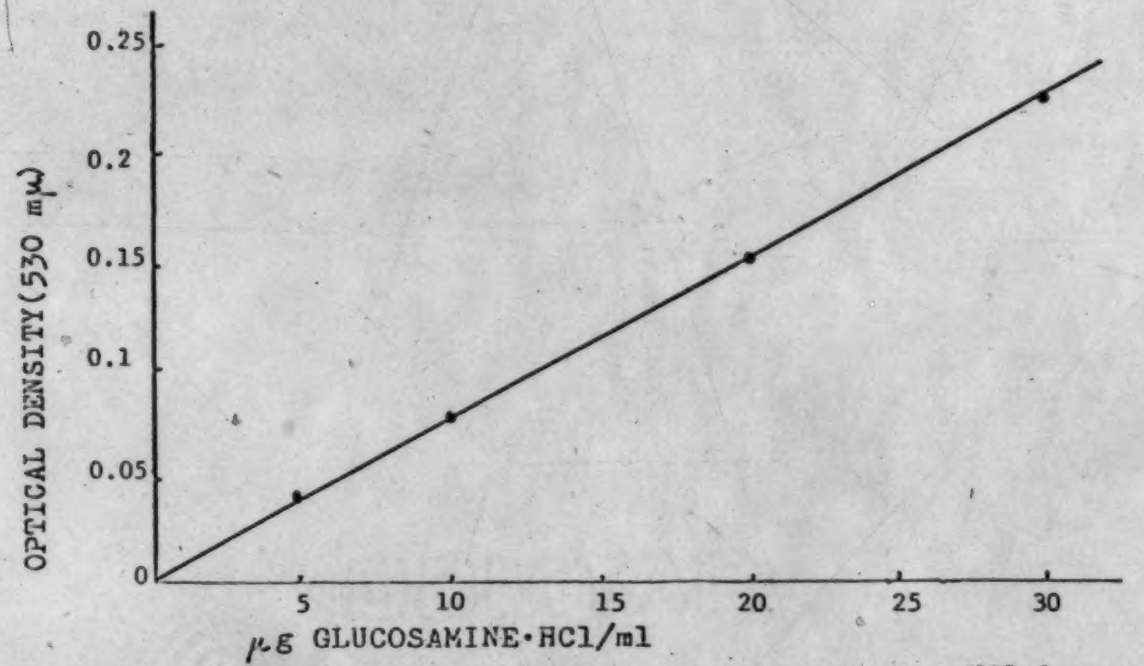



FIGURE 2 Optical density of reference glucosamine·HCl by Elson-Morgan reaction according to Boas(1953)(0.3 ml of 4M NaCl solution was added prior to the reaction).

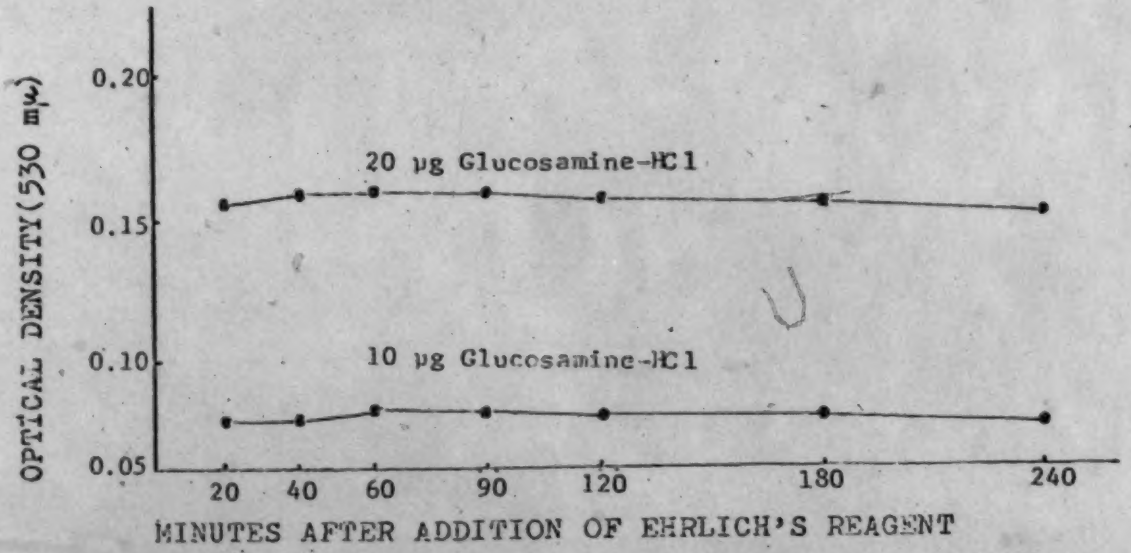



FIGURE 3 Stability of chromogen after addition of Ehrlich's reagent at room temperature.

addition of Ehrlich's reagent. The reproducibility of the method was excellent as the standard deviation of optical density expressed as a per cent of mean optical density in 4 different concentrations of glucosamine hydrochloride in 5 assays carried out during 3 different days ranged from 1.72 to 2.35%. It was decided that Boas' method was suitable for purified hexosamine analysis.

B. Quantitative Analysis of Total Hexosamine from Muscular Tissue Fractions

(a) Removal of Interfering Chromogens (Modification of Boas Method)

The main interfering substances in crude preparations are amino acids and carbohydrates which produce yellow or brown colored chromogens during acid hydrolysis. They also produce a red chromogen with acetylacetone and Ehrlich's reagent (Eastoe and Courts, 1963). This is a difficult problem numerous researchers have been struggling with for years (Pearce, personal communication).

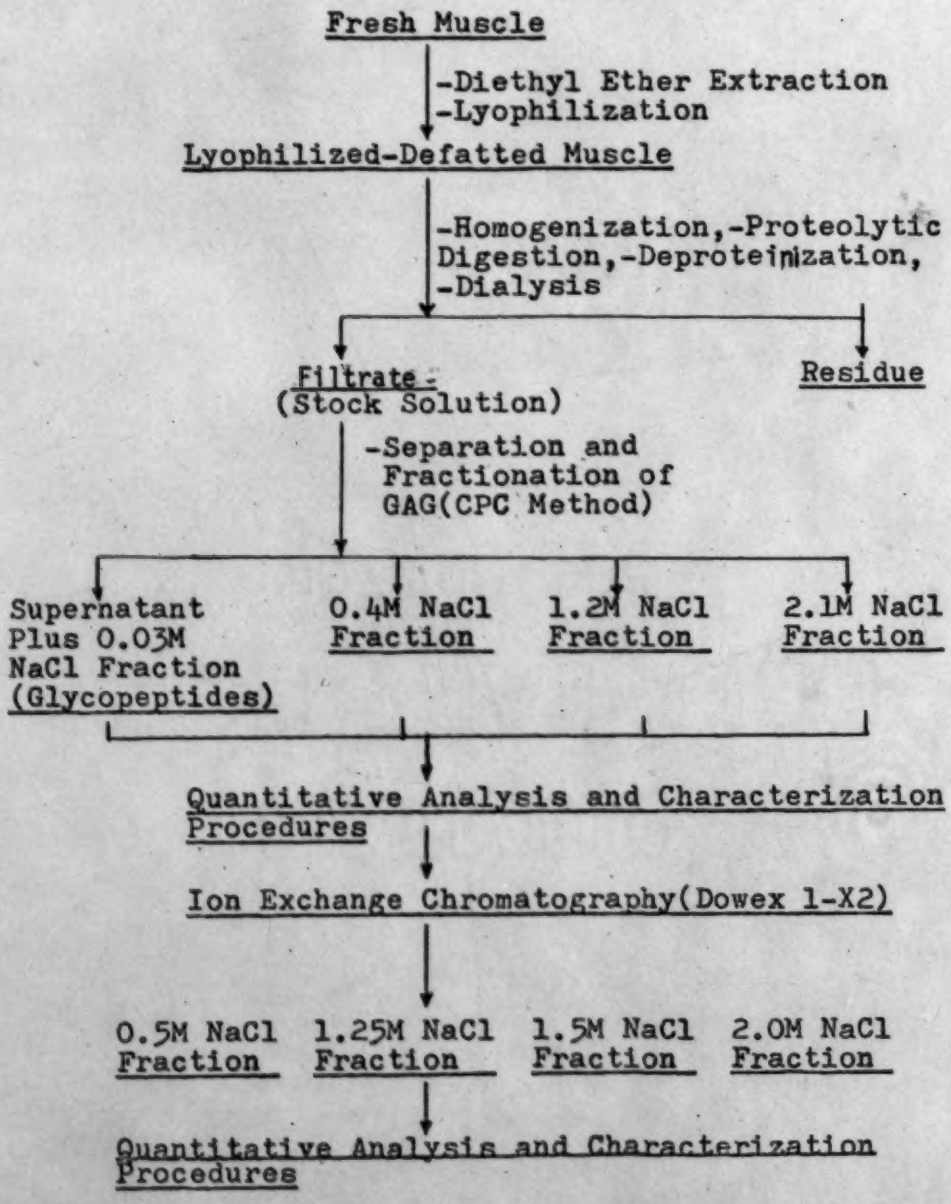
Boas (1953) used a cation exchange column (Dowex-50, 250-500 mesh) to remove interfering substances from tissue hydrolysates. Hexosamine was adsorbed on the resin, and then eluted with 2N HCl. In the case of muscle tissue, however, Boas (1953) could not accomplish complete removal of non-hexosamine chromogens. In this study, a Dowex-50W X 8 (200-400 mesh) column was tested with intramuscular tissue hydrolysates containing a glucosamine hydrochloride internal standard. Low internal standard recoveries, in the range of 60 to 70% were achieved. A dark band present at the top of the column may have retained some of the internal standard.

According to Vasseur and Immers (1949) hexosamines do not produce chromogens with Ehrlich's reagent when acetylacetone is omitted, whereas interfering substances do. Consequently a non-acetylacetone blank was introduced in this study, and the optical density of hexosamine was read against that of a non hexosamine chromophore from the non-acetylacetone blank. The recovery of internal standard glucosamine hydrochloride added to an intramuscular hydrolysate was found to be 97.9 to 101.4%. This method was therefore considered to be more suitable than the column chromatographic method of Boas (1953) for the analysis of crude samples for hexosamine determination.

(b) Sample Hydrolysis

Muscle glycopeptide and GAG fractions and proteolytic enzyme undigested residue fractions obtained from the TB muscle (see Figure 4) were hydrolyzed in 3N HCl in an autoclave at 121 C. The amount of hexosamine released as a function of hydrolysis time was measured using the above modification of Boas' method. The loss of internal standard glucosamine hydrochloride during hydrolysis was also measured. The hexosamine concentration was found to increase during the first 3 h of hydrolysis and thereafter slowly decrease as shown in Figure 5. A hydrolysis period of 3 h in length was therefore chosen. Five preparations of 20µg glucosamine hydrochloride were hydrolyzed under the above conditions. Mean optical densities were compared with those of non-hydrolyzed controls. The extent of destruction was found to be 3.4 ± 0.1% Eastoe and Courts (1963) found 13% of their glucosamine destroyed during hydrolysis in 4 N HCl at 100 C for 8 h. Ludowieg and Bennmaman (1967) observed 5.7% glucosamine and 4.2% galactosamine

Figure 4. Scheme for Extraction and Fractionation of GAG from Bovine-TB Muscle.



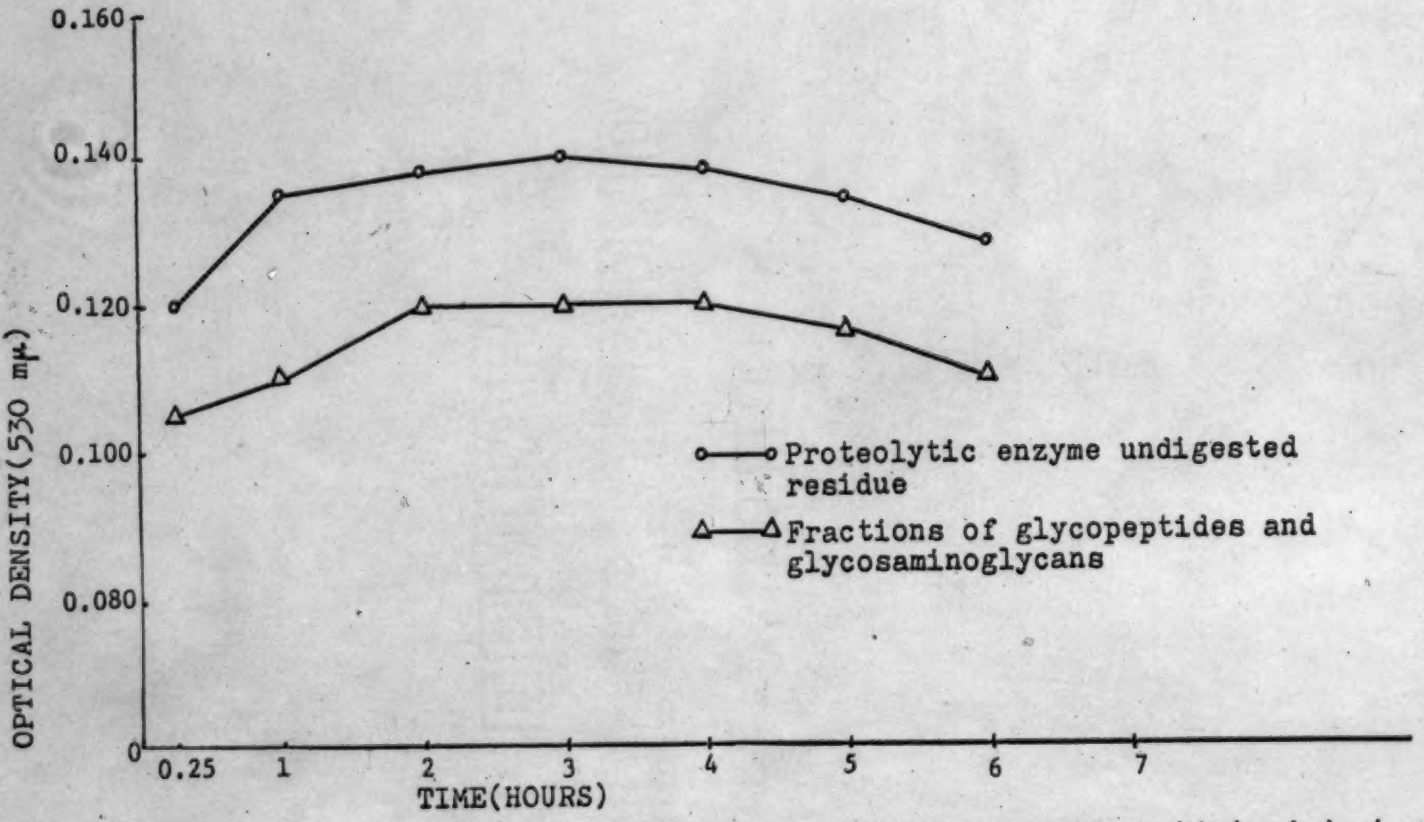



FIGURE 5 Optical density of chromogen formed when each fraction obtained during the GAG procedure was analyzed after hydrolysis in 3N HCl at 121 C for various lengths of time. Each value plotted was a mean from three determinations.

destroyed during hydrolysis in 4N HCl at 100 C for 14 h under nitrogen gas, and 50% glucosamine and 46% galactosamine destroyed during hydrolysis under vacuum in 6N HCl at 110 C for 22 h. According to Pearson (personal communication), when GAG preparations are hydrolysed, the per cent destruction of bound hexosamine is higher than that of free hexosamine. Therefore the value of 3.4% obtained in this study may be an underestimation of that occurring in the GAG fractions.

(c) Analysis of Total Hexosamine from Muscular Tissue Samples

Each muscle tissue fraction was hydrolyzed using the above described method. The hydrolysate produced from the proteolytic enzyme undigested residue obtained during GAG extraction was filtered through Whatman No. 1 filter paper before use. One ml of each sample containing 5 to 30 µg hexosamine was pipetted into each of 3 test-tubes with Teflon-lined screw caps. A water blank and standards using glucosamine hydrochloride were prepared in the same manner.

To each tube, 0.01 ml phenolphthalein reagent was added as the indicator, 4 N NaOH was added drop-wise until the sample just turned red and 0.5 N HCl was then added until the indicator colour just disappeared. Salt concentrations were made identical in all tubes with 4 N NaCl and volumes adjusted with water. One ml of 0.5 M sodium carbonate was pipetted into one tube and 1 ml of 2% (volume per volume) distilled acetylacetone in 0.5 M sodium carbonate added to the remaining two tubes. Each tube was tightly sealed and the sample acetylated in a water bath at 89 to 92 C for 45 min. The tubes were then cooled to room temperature, followed by the addition of 2.5 ml aldehyde-free ethanol and then 1 ml Ehrlich's reagent. After careful

shaking, aldehyde-free ethanol was added to bring the volume to 10 ml. One h after the addition of Ehrlich's reagent, the optical density was read at 530 mμ on a Beckman DB-G spectrophotometer. The difference in the optical density between acetylated and non-acetylated samples was used to calculate the concentration of hexosamine in each sample. A correction factor of 0.829 (the molecular weight ratio of glucosamine to glucosamine hydrochloride) was used to express values of the unknown in terms of free hexosamine.

3. Quantitative Analysis of Uronic Acid

The carbazole method (Dische, 1947), the modified carbazole method (Bitter and Muir, 1962) and the orcinol method (Brown, 1946; Khym and Doherty, 1952) have been extensively used to determine tissue levels of uronic acid. Each method gives a different color yield for the two kinds of uronic acid, glucuronic acid and iduronic acid. This can be a problem for quantitative analysis of the hybrid GAG (e.g., DS, HS and HP) containing both uronic acids. However, the different color yield obtained from each method is nevertheless useful for identifying the presence of each uronic acid. More recently, modifications improving the carbazole method have been reported by Galambos (1967) and Blumenkrantz and Asboe-Hansen (1973). Alternative uronic acid methods are documented elsewhere (Hanson et al., 1944; Dische, 1947a; Masamune and Aizawa, 1957; Helbert and Brown, 1961; Tracey, 1948). In this study, the modified carbazole method of Bitter and Muir (1962) was selected for the quantitative determination of uronic acid because of its relatively high sensitivity and routine reliability.

One-half ml of a sample containing 4 to 40 µg of uronic acid was placed in a test-tube and cooled on ice. Three ml of sulfuric acid reagent was slowly added while keeping the mixture on ice. The tube was then stoppered and heated in a boiling water bath for 10 min, followed by cooling to room temperature. One-tenth ml 0.125% carbazole in methanol was added; the tube was shaken well, heated in a boiling water bath for 15 min and cooled to room temperature. The optical density was read at 530 mµ with a Beckman DB-G spectrophotometer. When the uronic acid determination was carried out in the presence of cetylpyridium chloride (CPC) and NaCl, blanks containing the appropriate amounts of CPC and NaCl were prepared. The concentration of uronic acid was calculated by constructing a calibration curve using glucuronolactone as a standard.

4. Extraction of GAG

In a preliminary approach to this study, it was found that sequential digestion of muscular tissue with three different proteolytic enzymes, pepsin, papain and trypsin, resulted in maximum release of hexosamine (>90%). Duplicate homogenized-lyophilyzed-defatted samples (5g and 1g each of intramuscular and epimysial tissues, respectively, in Analysis I, and 25g and 5g each of intramuscular and epimysial tissues, respectively, in Analysis II) were digested by each of the following proteolytic enzyme preparations. Digestion by each enzyme was carried out within the same flask in the following order:

(1) 0.15mg pepsin (1:10,000) in 10 to 15ml of 0.1N acetic acid-HCl per g sample at pH 1.0 to 2.0 and 37 C for 12 h (Lowther et al., 1967).

(2) 0.03 g crude papain in 0.1M phosphate-0.005M disodium ethylenediamine tetraacetate-0.005M cystein hydrochloride per g sample at pH 6.5 and 65C for 16 h (Scott, 1963).

(3) 4 mg trypsin (1:250) per g sample at 37C for 12 h (Meyer et al., 1956) but at pH 7.0 instead of 7.5.

Five N NaOH was used to adjust the pH. Bacterial growth was inhibited by the addition of 0.1 ml 17 % benzalkonium chloride solution per 100 ml preparation (Fox, 1968; Wipf, 1969).

After proteolytic digestion, trichloroacetic acid (TCA) was added to a final concentration of 10 % by volume (Schiller et al., 1961), and the mixture was held at 3C overnight. The protein precipitate was removed by vacuum filtration through Whatman No. 50 filter paper with the aid of a 3 mm thick layer of celite. The celite layer was repeatedly washed with the 10 % TCA solution. The washings were pooled and quantitatively transferred into a dialysis bag. Dialysis was performed against running tap water at approximately 10C for 24 h, followed by several changes of distilled water at 3C for 24 h. The dialysate was quantitatively transferred into a 2 l round bottom evaporation flask and evaporated[1] to dryness under reduced pressure at 55C. After cooling the flask to room temperature, the dried residue was dissolved in 10 ml of water in Analysis I and 20 ml of water in Analysis II, transferred to a screw-cap plastic vial, tightly sealed and

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[1]Flash-Evaporator, Model FE-2, Buchler Instruments, Inc., Fort Lee, New Jersey.

stored at -30C as a crude GAG stock solution for subsequent analysis.

5. Recovery and Fractionation of GAG

Glycosaminoglycans are usually recovered by precipitation with a quaternary ammonium compound such as CPC or CTAB (Scott, 1963, 1965) which form quaternary ammonium-GAG complexes, or by precipitation with ethanol (Meyer et al., 1956). Cetylpyridinium chloride was used in this study (CPC method) because it is one of the most sensitive methods (Roden et al., 1972). Following the procedure of Schiller et al., (1961), cetylpyridinium-GAG complexes were produced, precipitated and fractionated by washing with different molarities of NaCl. Ion exchange column chromatography (Dowex 1-X2) was used to further fractionate the 1.2M NaCl fraction (Schiller et al., 1961)(see Figure 4). The principles of these fractionation methods are based on the different charge densities of the GAG.

Before application of these methods to the GAG extracted from muscle, preliminary tests were performed using standard GAG preparations:

(i) Preliminary Test with Reference GAG

A. CPC Method

Fifteen ml of each individual aqueous GAG reference solution containing 5 to 10 mg of a specific GAG (estimated by uronic acid analysis) were made to 0.035M NaCl. Three mg CPC were added per mg GAG to precipitate the GAG. After incubating for 1 h at 37C, 20 mg celite was added per mg GAG, the mixture was stirred thoroughly and centrifuged at 13000 X g and 22C for 20 min. The supernatant was decanted into another vessel. Additional washings were performed with 0.03M NaCl

in 0.1% CPC followed by centrifugation. The GAG-cetyl-pyridinium complexes obtained were fractionated by repeated washing with different molarities of salt; 0.4M NaCl in 0.1% CPC, 1.2M NaCl in 0.1% CPC and 2.1M NaCl. The supernatant from each wash was collected by centrifugation at 13000 X g and 22C for 10 min and was directly analyzed for uronic acid or galactose (see Section 6- vi) in the case of KS. If negligible uronic acid (or galactose) was detected, washing the precipitate with the particular NaCl solution was considered complete, and the molarity of salt was increased for separation of the next fraction.

The percentage of each standard GAG appearing in the three salt fractions is presented in Table 5. Hyaluronic acid was almost entirely recovered in the 0.4M NaCl fraction, while Ch4-S, Ch6-S, DS and HS were almost entirely recovered in the 1.2M NaCl fraction. These observations are in agreement with those of Schiller et al., (1961). Heparin, however, was recovered in two fractions, 53.3% in the 1.2M NaCl fraction and 44.7% in the 2.1M NaCl fraction. Schiller et al., (1961) reported 94 to 99% of their HP appeared in the 2.1M NaCl fraction. The distribution of KS was 68.2% in the 1.2M NaCl fraction and 20.1% in the 2.1M NaCl fraction. Schiller et al., (1961) found 58 and 42% in the 1.2 and 2.1M NaCl fractions, respectively. Chondroitin was not tested in this system because a commercial sample was not available. This GAG would likely be recovered in the 0.4M NaCl fraction (Anno et al., 1964; Davidson, personal communication).

Table 5. Per Cent Recovery of Reference GAG by the CPC Method

The amount of GAG extracted in each test tube was measured as uronic acid (or galactose in the case of KS) in the presence of NaCl and CPC[1]. The calculation of the per cent of each GAG recovered was based on the amount of each standard GAG added.

| | | Reference GAG[2] (% Recovery)[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Tube No. | Solvent | HA | Ch4-S | Ch6-S | DS | HS | HP | KS | Chs+HA |
| 1 | Supernatant | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0.03M NaCl in 0.1% CPC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3-8 | 0.4M NaCl in 0.1% CPC | 101.3 | 0 | 0 | 0 | 0 | 0 | 0 | (99.9)[4] |
| 9-13 | 1.2M NaCl in 0.1% CPC | 2.2 | 99.2 | 99.9 | 96.6 | 95.1 | 49.9 | 68.2 | (101.0)[5] |
| 14-16 | 2.1M NaCl | 0 | 2.6 | 2.3 | 3.5 | 5.6 | 48.9 | 20.1 | 2.1 |
| | Total Recovery | 103.5 | 101.8 | 102.2 | 100.1 | 100.7 | 98.8 | 88.3 | 103.0 |

Table 5. (continued)

[1]The effect of salts (NaCl and CPC) on the color yield of carbazol (or anthron reaction for galactose assay) was checked for HA in 0.4M NaCl containing 0.1% CPC, HA, DS, ChS, HS, HP and KS in 1.2M NaCl containing 0.1% CPC, and DS, ChS, HS, HP and KS in 2.1M NaCl. Approximately 37% and 40% color yield depression of HP compared to salt free control was found in 1.2M NaCl containing 0.1% CPC and 2.1M NaCl respectively. Approximately 10% increase of color yield of KS both in 1.2M NaCl containing 0.1% CPC and 2.1M NaCl compared to salt free control was observed. For other GAG, the salt effect was found to be small or negligible. Thus for the purpose of calculating per cent recoveries of HP and KS, the per cent color yield decrease or increase was taken into account.

[2]HS, HP and KS: Gifts from Dr. Mathews. Other GAG: from Sigma Chemical Co.

[3]Average values obtained from three trials.

[4]Per cent recovery of HA.

[5]Per cent recovery of ChS.

B. Column Chromatography of the 1.2M NaCl Fraction

One ml of an aqueous solution containing 5 to 10 mg individual standard GAG (estimated by uronic acid analysis) was applied to a 0.9 X 44 cm Dowex 1-X2 column[1]. The column was washed with water and the GAG eluted stepwise with increasing molarities of NaCl (0.5, 1.25, 1.5, 2.0 and 4.0M) at a rate of 10 to 12 ml per hr. Ten ml fractions were collected and directly assayed for uronic acid (or galactose in the case of KS). If negligible uronic acid (or galactose) was detected, the molarity of the salt was increased. This procedure was used in Analysis II only.

The per cent of each standard GAG eluted with various salt molarities is presented in Table 6. Hyaluronic acid, HS and ChS were almost entirely eluted with 0.5M, 1.25M and 1.5M NaCl respectively. These observations are in agreement with those of Schiller et al., (1961). Dermatan sulfate, from Sigma Chemical Co., was eluted to the extent of 75.5% with 1.5M NaCl and 23.0% with 2.0M NaCl, while a second dermatan sulfate, a gift from Dr. Mathews, was eluted to the extent of 55.3% with 1.5M NaCl and 40.1% with 2.0M NaCl. Although Schiller et al., (1961) did not study the elution of DS, other authors have reported the elution pattern of reference DS on a Dowex 1-X2 column. For example, Teller and Ziemann (1966) eluted DS entirely with 2.0M NaCl, while Bohn and Kalbhen (1971) eluted DS to the extent of 10.2, 26.0, 37.5 and 26.5% with 1.25, 1.5, 2.0, and 3.0M NaCl, respectively. The

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[1]See Appendix I for preliminary treatment of the resin.

Table 6. Per Cent Recovery of Reference GAG on a Dowex 1-X2 Column

The amount of GAG eluted in each test tube was measured as uronic acid (or galactose in the case of KS) in the presence of NaCl[1]. The calculation of the per cent of each GAG recovered was based on the amount of each standard GAG added.

| | | Reference GAG[2] (% Recovery)[3] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Tube No. | M NaCl | HA | ChS | DS-1 | DS-2 | HS | HP | KS |
| 1-13 | 0.5 | 97.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14-20 | 1.25 | 2.1 | 2.7 | 0 | 0 | 100.3 | 0 | 0 |
| 21-30 | 1.5 | 0 | 99.1 | 75.5 | 55.3 | 0 | 53.7 | 0 |
| 31-37 | 2.0 | 0 | 0 | 23.0 | 40.1 | 0 | 44.7 | 20.3 |
| 38-46 | 4.0 | 0 | 0 | 0 | 0 | 0 | 3.5 | 74.7 |
| Total Recovery | | 99.7 | 101.8 | 98.5 | 95.4 | 100.3 | 101.9 | 95.0 |

[1]The effect of salt on the color yield of carbazole (or anthron reaction for galactose assay) was checked for HA in 0.5M NaCl, HA, ChS and HS in 1.25M NaCl, ChS, DS and HP in 1.5M NaCl, DS, HP and KS in 2.0M NaCl, and HP and KS in 4.0M NaCl. Approximately 37%, 40% and 44% color yield depression of HP compared to salt free control was found in 1.5M, 2.0M and 4.0M NaCl respectively. Approximately 10% increase of color yield of KS both in 2.0 and 4.0M NaCl compared to salt free control was observed. For other GAG, the salt effect was found to be

Table 6. (continued)

small or negligible. Thus for the purpose of calculating per cent recoveries of HP and KS, the per cent color yield decrease or increase was taken into account.

[2]HS, HP, KS and DS-2: from Dr. Mathews. Other GAG: from Sigma Chemical Co.

[3]Values based on one trial.

elution distribution of HP in this study was 53.7% with 1.5M NaCl, 44.7% with 2.0M NaCl and 3.5% with 4.0M NaCl. Bohn and Kalbhen (1971) reported that HP was eluted 2.5, 40.5, 49.0 and 8.0%, with 1.25, 1.5, 2.0 and 3.0M NaCl respectively, while Schiller et al., (1961) and Teller and Ziemann (1966) eluted all HP with 2.0M NaCl. Keratan sulfate, in this study, was eluted to the extent of 20.3% with 2.0M NaCl and 74.7% with 4.0M NaCl, similar to the approximately 19% with 2.0M NaCl, 78% with 3M NaCl and 3% with 4M NaCl reported by Schiller et al., (1961). Whereas Teller and Ziemann (1966) eluted all KS with 3.0M NaCl and no KS was eluted with a lower molarity of salt than 3.0M. Bohn and Kalbhen (1971) reported a wide range of elution patterns of KS from 1.25 to 3.0 M NaCl. These different elution patterns of DS, HP and KS may be due to the heterogeneity of the GAG and differences in preparation techniques. Chondroitin would likely be eluted with 0.5M NaCl on a Dowex 1-X2 column (Seno, 1972).

(ii) Isolation and Fractionation of Muscule GAG

The crude GAG stock solution prepared as outlined in section VI-4 was made to 0.035M NaCl and 3 mg CPC was added per mg GAG (estimated by hexosamine analysis) to precipitate the GAG. The fraction which does not precipitate with CPC was called the glycopeptide fraction (Lowther et al., 1967), which was stored frozen at -30C for subsequent analysis. The precipitated GAG was fractionated as outlined previously for the reference GAG.

In the 1.2M NaCl fraction of Analysis I and in the 0.4, 1.2 and 2.1M NaCl fractions of Analysis II, which were characterized in considerable detail, CPC was removed by precipitation with potassium

thiocyanate (Korn, 1959). The supernatant containing GAG was filtered through Whatman No. 1 filter paper, dialyzed and concentrated by lyophilization. In Analysis II, further separation of the component GAG in the 1.2M NaCl fraction was carried out on a Dowex 1-X2 column as outlined previously for the reference GAG. The other fractions obtained were stored at -30C for the subsequent characterization methods.

6. Characterization of GAG

In both Analyses I and II, each GAG fraction was characterized by the determination of its component; uronic acid, hexosamine, sulfate and hexose, and by calculation of its molar ratio of uronic acid to hexosamine. In addition, each GAG fraction was characterized by enzymatic methods using testicular hyaluronidase, chondroitinases and sulfatases. In Analysis II, each GAG fraction was further characterized by electrophoretic methods.

(i) Uronic Acid Analysis

The following three methods were used for the analysis of uronic acid:

(1) The original carbazole method (Dische, 1947).

(2) The modified carbazole method (Bitter and Muir, 1962), as outlined in Section VI-3.

(3) The orcinol method (Brown, 1946; Khym and Doherty, 1952).

In the case of iduronic acid, the color yield obtained in the Dische procedure is approximately 50% of that obtained in the Bitter and Muir procedure (Toole and Lowther, 1966). In the orcinol reaction, the color yield of glucuronic acid is about 85% of that of iduronic acid due to

the incomplete liberation of D-glucuronic acid (Roden et al., 1972). Used in conjunction with each other, the original and modified carbazole methods and orcinol method can provide information concerning the relative proportions of glucuronic acid and iduronic acid in a mixture of GAG. For example, the carbazole:orcinol color yield ratio of iduronic acid is 0.23 and in DS which almost exclusively contains iduronic acid, the ratio value is close to 0.23 (Roden et al., 1972).

For the original carbazole method, which was carried out according to Dische (1947), 0.5 ml sample containing 5 to 100 µg uronic acid was used. For the orcinol method, which was carried out according to Brown (1946) and Khym and Doherty (1952), 3 ml of orcinol reagent were added to 1.0 ml of sample containing 2 to 40 µg uronic acid, and heated in a capped tube in boiling water for 30 min. The color developed was measured at 660 mµ in a Beckman DB-G spectrophotometer.

(ii) Molar Ratio of Uronic Acid to Hexosamine

The values of uronic acid determined by the carbazole reaction (Bitter and Muir, 1962) and hexosamine determined by the modified Boas (1953) technique were used to calculate the molar ratio of uronic acid to hexosamine.

(iii) Sulfate Analysis

The extent of sulfation of the GAG fractions was determined by the method of Terho and Hartiala (1971). Sodium sulfate was used as a standard. A linear standard curve was obtained between 1 and 10 µg $Na_2SO_4$ per 0.5 ml water. The color formed was stable for 30 min following addition of an 80% ethanol solution containing 0.005%

sodium rhodizonate and 0.1% ascorbic acid. After 45 and 60 min, the color intensity was depressed 2 and 8% respectively.

(iv) Hyaluronidase Digestion

Testicular hyaluronidase, an endo β-hexosaminidase[1], hydrolyzes the β-hexosamidic bond between D-glucuronic acid and hexosamine found in HA, Ch and ChS. Heparin and HS which have α-hexosamidic bonds between the two constituent sugars, DS which has glucuronic acid instead of iduronic acid, and KS which has no uronic acid, are not affected by this enzyme (Suzuki, 1969). Therefore the test by hyaluronidase digestion will provide evidence as to whether or not testicular hyaluronidase susceptible GAG are present in preparations.

Glycosaminoglycan fractions were incubated with testicular hyaluronidase according to the method of Suzuki (1969). The amounts of undegraded GAG were determined by a modified form of the turbidimetric method of DiFerrante (1956).

Four tubes in duplicate were used for the analysis of each sample. Tube (1) contained a mixture of 0.3 ml of sample containing 80 to 150 µg GAG estimated by uronic acid analysis, 0.3 ml phosphate-citrate buffer (pH 5.0), and 0.4 ml hyaluronidase (32 to 60 National Formulary Units) in 1M NaCl. Tube (2), the GAG free control of tube (1), contained 0.3 ml water in place of 0.3 ml sample and other components identical to tube (1). Tube (3), hyaluronidase free preparation of tube (1), contained 0.4 ml 1M NaCl free of hyaluronidase and other components identical to

[1]Testicular hyaluronidase also acts as transglycosidase (Suzuki, 1969).

tube (1). Tube (4), the GAG free control of tube (3),contained 0.3 ml water and 0.4 ml 1M NaCl and 0.3 ml phosphate-citrate buffer. These four tubes were screw-capped and incubated at 37C for 16 h. Two ml of CTAB reagent were then added and the solution thoroughly mixed. The resulting turbidity was read within 10 min at 400 mμ on a Beckman DB-G spectrophotometer. The testicular hyaluronidase digestibility in each sample was calculated as the per cent fall in turbidity as follows:

$$(1 - T_A/T_B) \times 100\%$$

where $T_A$ = turbidity reading of tube (1) against tube (2);

$T_B$ = turbidity reading of tube (3) against tube (4).

(v) Enzymatic Assay of the Isomeric Galactosaminoglycans

A. Introduction

Since it is very difficult to separate the isomeric galactosaminoglycans, Ch4-S, Ch6-S and DS by either the CPC method or by ion-exchange chromatography (Suzuki, 1969), enzymatic analysis was performed according to Saito et al., (1968) and Yamagata (1969).

The four enzymes used in this analysis were chondroitinase-ABC, chondroitinase-AC, chondro-4-sulfatase, and chondro-6-sulfatase. The reactions they catalyze are as follows:

(1) Ch4-S $\xrightarrow{\text{chondroitinase-ABC}}$ nΔDi-4S[1]

(2) DS $\xrightarrow{\text{chondroitinase-ABC}}$ nΔDi-4S

(3) Ch6-S $\xrightarrow{\text{chondroitinase-ABC}}$ nΔDi-6S[2]

(4) Ch4-S $\xrightarrow{\text{chondroitinase-AC}}$ nΔDi-4S

(5) Ch6-S $\xrightarrow{\text{chondroitinase-AC}}$ nΔDi-6S

(6) ΔDi-4S $\xrightarrow{\text{chondro-4-sulfatase}}$ nΔDi-OS[3] + $SO_4^{--}$

1, 2, 3See list of Abbreviations.

(7) ΔDi-6S $\xrightarrow{\text{chondro-6-sulfatase}}$ nΔDi-0S + $SO_4^{--}$

where n indicates the number of repeating units.

It has been reported (Saito et al., 1968; Yamagata, 1969) that chondroitinase-ABC cleaves the acetylhexosamidic bond attaching the C-4 position of the D-glucuronic acid residue or L-iduronic acid residue in Ch4-S, Ch6-S and DS. It cleaves the same bond in Ch and HA, though relatively more slowly. In both cases, the 4,5-unsaturated disaccharides (ΔDi-4S and Di-6S) are produced. Chondroitinase-ABC does not attack KS, HS and HP. Chondroitinase-AC does not react with DS, but carries out essentially the same reactions as chondroitinase-ABC with Ch4-S, Ch6-S, Ch, and HA. Chondro-4-sulfatase and chondro-6-sulfatase carry out hydrolytic desulfation of the chondroitinase enzyme digested products, ΔDi-4S and ΔDi-6S respectively, to produce ΔDi-0S, and do not attack polymeric GAG (Yamagata et al., 1968; Suzuki, 1969). The amounts of unsaturated disaccharides, ΔDi-0S and ΔDi-6S liberated can be measured colorimetrically by the Morgan-Elson raction (Saito et al., 1968).

B. Preliminary Analysis

In a preliminary analysis, known amounts of individual reference GAG (Ch4-S, DS, Ch6-S, and HA) were measured as uronic acid and known mixtures of these same GAG were analyzed by the enzymatic method. The recovery of each GAG as uronic acid was compared with values obtained by the enzymatic method. In the case of DS, a factor 0.807 (optical density ratio, DS:Ch4-S, Bitter and Muir, 1962) was used to correct for its lower color yield in the carbazole method. The amounts of individual GAG recovered were 104.5% for Ch4-S, 102.0% for DS and 97.0%

for Ch6-S. For a mixture of Ch4-S:DS:Ch6-S in a molar ratio of 3.0: 3.0: 4.0, the recovered ratio was 3.0: 2.9: 4.2, and for a mixture of Ch4-S : DS : Ch6-S : HA in a molar ratio of 3.0 : 3.5 : 3.0 : 0.5, the rocovered ratio was 3.1 : 3.2 : 3.1 : 0.5. Reactions of the four enzymes used in this section of the study were not found to be inhibited by HS, HP or KS. These enzymes were also tested in the presence of 0.1% CPC, and were found to be strongly inhibited by CPC. Consequently it was essential to completely remove CPC from the assay solutions.

C. Application of the Enzymatic Technique to Tissue Samples

For each GAG sample, five tubes were prepared containing 15 µl of each of the solutions listed below:

Tube (1): substrate and tris buffer;

Tube (2): substrate, tris buffer, chondroitinase-ABC and chondro-6-sulfatase;

Tube (3): substrate, tris buffer, chondroitinase-AC, chondro-6-sulfatase and chondro-4-sulfatase;

Tube (4): substrate, tris buffer, chondroitinase-ABC, chondro-4-sulfatase and chondro-6-sulfatase;

Tube (5): substrate, tris buffer and chondroitinase-ABC.

Water was added to Tubes 1, 2 and 5 to bring to a total volume of 75 µl. The tubes were screw-capped and incubated at 37C for 30 min. After incubation, the Morgan-Elson reaction was carried out in each tube according to Saito et al., (1968). The optical density was read at 585 mµ in a Beckman DU spectrophotometer, and the concentration of each isomer was calculated.

In a preliminary test, muscle preparations were found to contain large amounts of DS. It has been reported by Saito et al., (1968) that DS interferes with the reaction of chondroitinase-AC with Ch4-S and Ch6-S; therefore care must be taken that adequate amounts of chondroitinase-AC are used. Consequently in Analysis II, galactosaminoglycan samples were digested with hyaluronidase in a sixth tube as suggested by Saito et al., (1968), since this enzyme is not known to be inhibited by DS. Following incubation of tube (6), the DS which could not be digested with hyaluronidase was converted to ΔDi-OS by chondroitinase-ABC and chondro-4-sulfatase. Analytical values obtained from hyaluronidase digestion and chondroitinase-AC digestion for mixtures of isomeric galactosaminoglycans, were found to be similar, suggesting that the amount of chondroitinase-AC used in both Analyses I and II was adequate.

(vi) Hexose Analysis

Hexose was determined by the anthron reaction according to Trevelyan and Harrison (1952) using galactose as the standard hexose. This method was used for the purpose of detecting KS which contains galactose in place of uronic acid.

(vii) Electrophoresis of GAG

Glycosaminoglycans are known to have either a sulfate or a carboxyl group, or both. The amounts and positions of these groups affect the charge density of each GAG. On the basis of their different charge densities, GAG can be readily separated and identified by electrophoresis. One dimensional electrophoresis was carried out on cellulose acetate membranes using three different solvent systems:

(1) Phosphate buffer solution at pH 6.7, with a constant current of 1 mA per cm for 1 h (Hata and Nagai, 1972);

(2) 0.3M calcium acetate solution at pH 7.25, with a constant current of 1 mA per cm for 3 h (Seno et al., 1970);

(3) 0.1M barium acetate at pH 8.0, with a constant current of 1 mA per cm for 4.5 h (Hata and Nagai, 1972).

Initially solvent system (1) was used for separation of all GAG fractions; however the relative mobilities of the isomeric galactosaminoglycans, Ch4-S, Ch6-S and DS, were identical. To separate these isomers, solvent system (2) was employed. Solvent system (3) was used to obtain improved separation of the sulfated GAG in the 2.1M NaCl fraction.

Each GAG fraction was concentrated by lyophilization and 0.5 to 2.0 µg in 0.5 µl was applied to 5.5 X 14 cm cellulose acetate membranes[1] which had been previously rinsed in their appropriate solvent system. Standard GAG were spotted on each cellulose acetate membrane and electrophoresis was performed at room temperature. Following electrophoresis, the membranes were stained to identify each GAG. Membranes which were run in phosphate buffer or in barium acetate solvent system were stained with 1% alcian blue in 0.1% acetic acid for 10 min, washed with 0.1% acetic acid for 20 min, and dried between filter papers (Hata and Nagai, 1972; Hata, 1973). Membranes from the calcium acetate solvent system were stained with 0.5% alcian

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[1] Beckman Instruments Inc., Fullerton, California.

blue in 3% acetic acid for 20 min and washed with 1% acetic acid for 1 min.

The relative amount of each GAG isomer was determined by colorimetric or densitometric methods. For the colorimetric method, membranes were washed with tap water for 10 min, after which each GAG spot was removed along with an adjacent GAG free portion of the cellulose acetate membrane of equal area to act as a background. Each portion of the membrane was placed in separate test-tubes containing 1.0 ml of 5% CPC solution. The tubes were capped and heated in boiling water for 15 min. The dye bound to the GAG was extracted by the CPC solution, and the optical density of the solution was read against the background solution with a Beckman DU spectrophotometer at 615 mμ. The proportion of each isomer was calculated from the optical density of each spot. For the densitometric method, each membrane was soaked in 96% ethanol for 30 sec, glacial acetic acid : ethanol solution (1:3) for 1 min, pressed on a glass board and dried in an air circulating oven at 100C for 10 min. The dried electrophoretogram was kept in a plastic holder, and the position and relative density of isomers were scanned at 600 mμ by a densitometer[1] using 0.1 X 2 mm slit width at a scanning speed of 1 in per min and a chart speed of 4 in per min. The proportion of each isomer was calculated from its peak area. The colorimetric method used was essentially that of Hata (1973), while the densitometric method used was essentially that of Seno et al., (1970).

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[1]Photovolt Transmission Densitometer, Model 52C, Photovolt Corporation, New York City.

(viii) Glucosamine and Galactosamine Analysis

A. Preliminary Approach

The method of Gardell (1953) was found to give a clear separation of glucosamine and galactosamine by Dowex-50W X 8 (200-400 mesh) ion exchange chromatography. The only problem with this method was that it was too time consuming, approximately 50 h for each sample.

Gas liquid chromatography has been reported by Sweeley et al., (1963) to give a rapid and quantitative separation of glucosamine and galactosamine. Figure 6 shows several gas chromatograms which were run with trimethylsilyl derivatives of reference hexosamines and hexosamines from reference GAG. In this method, incomplete dissolution of hexosamine in the solvent was found to be a problem; however it was considered that the method was useful for qualitative analysis of the major hexosamine present in each GAG fraction.

B. Glucosamine and Galactosamine Analysis by Gas Liquid Chromatography

Samples from the 0.4M NaCl and 1.2M NaCl fractions obtained from the CPC method and condensed by lyophilization were subjected to glucosamine and galactosamine analysis by gas liquid chromatography. The procedure was performed at 175C with a gas chromatograph[1] using a 5 ft glass column (4 mm i.d.) packed with 3% OV-1 on 80-100 mesh Gas Chrom Q[2]. Recorder chart speed was 1 in per min.

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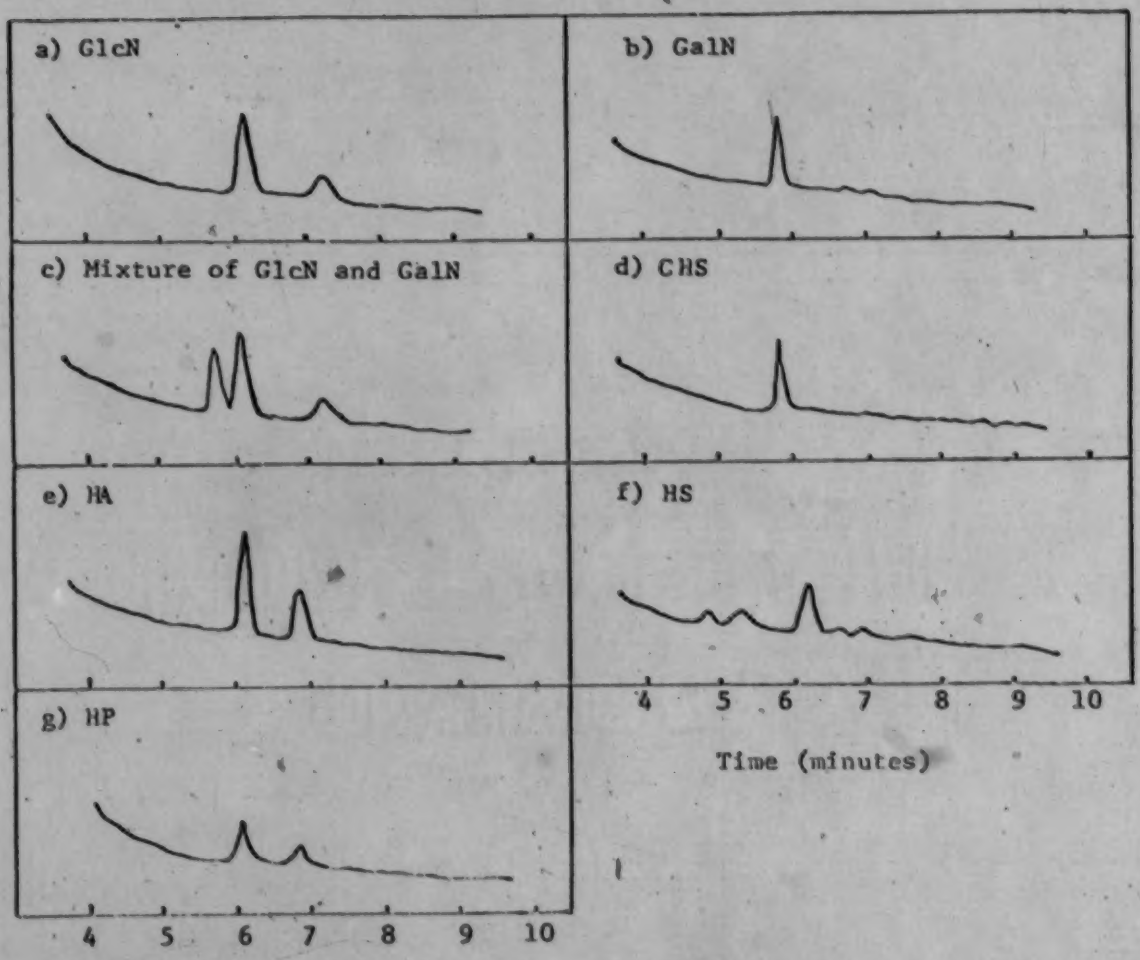
[1]Bendix Gas Chromatograph 2500, Bendix Process Instruments, Ronceverte, West Virginia.

[2]Applied Science Laboratories, Inc., State College, Pennsylvania.

FIGURE 6 Analysis of Trimethyl Derivatives of Reference Hexosamines and Hydrolyzed GAG by Gas Liquid Chromatography.




1.CHS:Sigma Chemical Co. and Others:Gifts from Dr.M.B.Mathews.

Each sample containing 100 to 500 µg hexosamine was placed in a 50 ml test tube with a Teflon lined screw cap, hydrolysed in 3N HCl at 121C for 3 h and evaporated to dryness in a vacuum oven at 55C. The trimethylsilylated derivatives were produced by heating 100 µg of hexosamine with 20 µl pyridine, 4 µl hexamethyldisilazane, and 2 µl trimethylchlorosilane in a water bath for 3 min at 85C. One to 3 µl of the reaction solution was injected onto the column. The relative amounts of glucosamine and galactosamine in each sample were determined by comparing the peak areas.

7. Additional Analyses of GAG Fraction

Quantitative analyses of protein and sialic acid, and qualitative analysis of uronic acid were performed in Analysis II.

(i) Quantitative Analysis of Protein

The protein concentration was monitored throughout the GAG isolation procedure and the amount of protein contaminating the GAG preparations was determined. The method of Lowry et al., (1951) was used.

(ii) Quantitative Analysis of Sialic Acid

The thiobarbituric acid method of Warren (1959) was used for the analysis of sialic acid. This method was considered to give a more precise estimation than other methods such as the direct Ehrlich reaction (Werner and Odin, 1952) or the resorcinol reaction (Svennerholm, 1956). N-acetyl-neuraminic acid was used as a standard.

(iii) Qualitative Analysis of Uronic Acid

Qualitative analysis of uronic acid was carried out to ascertain

whether or not there were unprecipitated GAG in the glycopeptide fractions (see Figure 4). The thin-layer chromatographic method of Stahl and Kaltenbach (1965) was used. A known amount of the glycopeptide fraction, measured as hexosamine, was hydrolyzed in 1N HCl at 100C for 5h, evaporated in a vacuum oven at 55C and diluted with water (Kondo et al., 1971). Five to 50 µl of each preparation containing 500 to 1000 µg of hexosamine was spotted on a 20 X 20 cm Silica Gel-G layer[1] (soaked in 0.1N boric acid and dried prior to use) with 5 to 50 µg of reference glucuronolactone and galactose. Chromatography and subsequent detection of uronic acid were performed according to Stahl and Kaltenbach (1965).

## VII. DATA ANALYSIS

All data, other than that in Analysis II, were subjected to analysis of variance (Steel and Torrie, 1960). Duncan's multiple range test (Duncan, 1955) was used to determine differences among means. All tests were done at the 5% level of significance. All the data were calculated on a within group (age and sex) basis. Data obtained in Analysis II were not subjected to statistical analysis.

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[1] Pre-coated TLC-plate SiLG-25 UV 254. Machery-Nagel Co., Werkstrasse, Germany.

## RESULTS AND DISCUSSION

### I. HISTOCHEMICAL ANALYSIS OF BOVINE TB MUSCLE

#### 1. Results

Although not measured, it was apparent by microscopic observations that muscle fiber diameter increased until the animals became yearlings; thereafter myofiber diameters appeared similar for the remaining older age and sex groups studied. Epimysial tissue thickness was observed to increase with advancing animal age, and was observed to be much thicker in males than in females of comparable ages. These values were also not recorded.

Collagen, elastin, reticulin and MPS histochemical scores, along with values for the ratio of the collagen score : reticulin score and MPS score : collagen score are presented in Table 7. Results obtained from histochemical analysis of animals of similar ages in Trials I and II are similar and show similar trends.

#### (i) Collagen Scores

Fetal intramuscular collagen scores were significantly higher than post natal intramuscular collagen scores. In contrast, fetal epimysial collagen scores were significantly lower than post natal scores. The age related decrease of post natal intramuscular collagen scores is likely due to the rapid myofiber hypertrophy in fetal and young post natal animals up to the age of the yearlings. The lower fetal epimysial collagen scores possibly reflect dilution by the larger amounts of reticulin and MPS found in fetal epimysial

Table 7. Mean Histochemical Scores of Bovine TB Connective Tissue

| Age and Sex Group | Mean Histochemical Score | | | | | |
|---|---|---|---|---|---|---|
| | Collagen | Elastin | Reticulin | Ratio Collagen: Reticulin | MPS | Ratio MPS: Collagen |
| **TRIAL I** | | | **Intramuscular Tissue** | | | |
| FI | $6.20^{a}$ | $2.00^{a}$ | $5.70^{a}$ | $1.10^{a}$ | $5.90^{a}$ | $0.95^{a}$ |
| FII | $5.87^{a}$ | $1.87^{a}$ | $5.67^{a}$ | $1.03^{a}$ | $5.00^{b}$ | $0.85^{b}$ |
| I | $2.50^{b}$ | $1.30^{a}$ | $3.43^{b}$ | $0.75^{b}$ | $1.85^{c}$ | $0.74^{c}$ |
| IIf | $2.00^{b}$ | $1.33^{a}$ | $2.83^{c}$ | $0.73^{b}$ | $1.45^{c}$ | $0.73^{c}$ |
| IIm | $2.05^{b}$ | $1.25^{a}$ | $2.79^{c}$ | $0.73^{b}$ | $1.40^{c}$ | $0.68^{c}$ |
| III | $2.08^{b}$ | $1.50^{a}$ | $2.75^{c}$ | $0.74^{b}$ | $1.41^{c}$ | $0.68^{c}$ |
| | | | **Epimysial Tissue** | | | |
| FI | $5.50^{a}$ | $3.00^{a}$ | $5.39^{a}$ | $1.10^{a}$ | $7.00^{a}$ | $0.96^{a}$ |
| FII | $5.93^{b}$ | $3.00^{a}$ | $5.01^{b}$ | $1.17^{a}$ | $6.60^{b}$ | $0.84^{b}$ |
| I | $7.00^{c}$ | $3.06^{a}$ | $2.93^{c}$ | $2.41^{b}$ | $3.67^{c}$ | $0.52^{c}$ |
| IIf | $7.00^{c}$ | $2.50^{a}$ | $2.23^{d}$ | $3.14^{c}$ | $3.25^{cd}$ | $0.46^{cd}$ |
| IIm | $7.00^{c}$ | $3.25^{a}$ | $2.00^{d}$ | $4.08^{cd}$ | $3.25^{cd}$ | $0.46^{cd}$ |
| III | $7.00^{c}$ | $3.13^{a}$ | $1.33^{d}$ | $5.65^{d}$ | $2.50^{d}$ | $0.36^{d}$ |
| **TRIAL II** | | | **Intramuscular Tissue** | | | |
| IIf | $2.60^{a}$ | $1.50^{a}$ | $2.25^{a}$ | $1.15^{a}$ | $2.00^{a}$ | $0.77^{a}$ |
| IIm | $2.33^{a}$ | $1.17^{a}$ | $2.33^{a}$ | $1.00^{ab}$ | $2.00^{a}$ | $0.86^{a}$ |
| III | $2.30^{a}$ | $1.50^{a}$ | $3.00^{a}$ | $0.70^{b}$ | $2.00^{a}$ | $0.87^{a}$ |
| | | | **Epimysial Tissue** | | | |
| IIf | $7.00^{a}$ | $2.33^{a}$ | $2.07^{a}$ | $3.40^{a}$ | $2.67^{a}$ | $0.38^{a}$ |
| IIm | $7.00^{a}$ | $2.33^{a}$ | $1.97^{a}$ | $3.55^{a}$ | $2.67^{a}$ | $0.38^{a}$ |
| III | $7.00^{a}$ | $3.00^{a}$ | $1.95^{a}$ | $3.59^{a}$ | $2.50^{a}$ | $0.36^{a}$ |

a,b,c,d - Means in the same vertical row, within a tissue, with different superscripts are significantly different ($P<0.05$).

tissues. The intramuscular collagen score did not change during the fetal growth period studied, while the epimysial value decreased significantly during the fetal growth period. No significant differences in the collagen score were found in either the post natal intramuscular or the post natal epimysial groups. Post natal epimysial collagen scores were found to be approximately three times higher than post natal intramuscular scores.

(ii) Reticulin Scores

Both intramuscular and epimysial reticulin scores were significantly higher in the fetal groups than in the yearling groups which in turn had a significantly higher score than the adult groups. The reticulin scores for all adult groups were similar. In the intramuscular tissue, only a slight response of reticulin was observed in the perimysium in the yearling groups, while an almost negligible reticulin response was observed in the perimysium in the remaining adult groups. However the endomysium displayed a reticulin positive response in all the age and sex groups studied. Intramuscular reticulin scores were slightly higher than epimysial scores from tissues of animals of comparable ages.

(iii) Collagen Score : Reticulin Score Ratios

The collagen score : reticulin score ratios were calculated as an estimate of the relative amount of these two connective tissue proteins. The intramuscular ratio values were found to be significantly higher in both fetal groups than in the post natal groups. The collagen : reticulin score ratios were similar among all

post natal groups, while the epimysial ratios were found to increase with age. No significant sex effect was observed in either tissue. All fetal intramuscular and epimysial ratio values were found to be similar, while the post natal ratio values of intramuscular tissue were found to be considerably lower than those of the epimysial tissue.

(iv) Elastin Scores

A relatively limited, though constant amount of elastin was found in both intramuscular and epimysial tissues. The elastin was found largely in the blood vessels of the perimysial and epimysial tissues. The elastin score was found to be consistently two to three times higher in the epimysial tissues than in the intramuscular tissues from animals of comparable ages.

(v) Mucopolysaccharide Scores

Both the intramuscular and the epimysial MPS scores appeared to continually decrease with increasing animal age. The MPS scores of the younger fetal samples were significantly higher than those of the older fetal samples for both intramuscular and epimysial tissues. These fetal MPS scores were in turn significantly higher than those of all post natal MPS scores. Within the post natal groups, there was a gradual decline in the MPS score with increasing animal age. This decline was not as great in the intramuscular tissues as in the epimysial tissues, in which the mean mature adult MPS score was found to be significantly lower than the mean yearling score. The MPS score was found to be consistently lower in intramuscular tissue than

in epimysial tissue from animals of comparable ages.

(vi) Mucopolysaccharide Score : Collagen Score Ratios

The MPS score : collagen score ratios were calculated to provide an indication of the amount of ground substance relative to collagen in each sample. The values for the ratio in fetal intramuscular and fetal epimysial tissues were found to be similar. However, the scores from the younger fetal group were significantly greater than the scores from the older fetal group. The values of the ratio for the fetal tissues were, in all cases, significantly higher than the values for the post natal tissues. In post natal tissues, the intramuscular MPS : collagen ratios were consistently higher than the epimysial ratios, indicating the presence of a relatively greater amount of ground substance than collagen in the intramuscular than in the epimysial tissue.

2. Discussion

Information concerning age and sex associated histochemical changes in muscle is limited. An increase of epimysial thickness with advancing age and thicker epimysial tissues in young adult males than in young adult females were observed in this study. Similar observations have been reported for the increased epimysial thickness with advancing age in porcine muscle (Carpenter et al., 1963) and for the thicker LD epimysial tissue in bulls than in steers of similar age (Field et al., 1969).

The significance in the fetal collagen score is due to relatively smaller myofiber mass in the intramuscular tissue and higher scores of

reticulin and MPS in the epimysial tissue. The reticulin response of endomysial samples observed in this study is supported by the recent report of Swatland (1975) who demonstrated endomysial reticular fibers in porcine and bovine muscles. Observations of the epimysial collagen score : reticulin score ratio are consistent with that of Gross (1950) who studied rat skin histochemically and observed an increased collagen and a decreased reticulin staining reaction with increasing animal age. The lower post natal collagen : reticulin score ratios in intramuscular tissues than in epimysial tissues are likely due to the reticulin response of endomysial tissues which make up the majority of the intramuscular connective tissue. Elastin was found to be a relatively minor component of bovine TB muscle. This observation is consistent with the observation of Vognarová et al., (1968) that elastin is a minor component of skeletal muscle.

The significantly higher MPS score in fetal tissue reflects a relatively greater amount of ground substance in this tissue. Analysis of hexosamine and uronic acid reported later in this thesis confirms this observation. The observed decrease of the MPS score : collagen score ratio with increasing age is consistent with that of Gross and Schmidt (1948), who studied human abdominal skin from individuals ranging in age from one hour to 89 years and reported that with advancing age, the proportion of fibrous components increases, whereas that of ground substance decreases.

The author has not found information in the literature concerning the histochemical report of the overall age and sex associated changes in skeletal muscle connective tissue.

## II. BOVINE TB NITROGEN, COLLAGEN AND LABILE FRACTIONS

### 1. Intramuscular and Epimysial Nitrogen and Collagen Concentrations

#### (i) Results

In both Trials I and II, nitrogen and collagen concentrations were determined in both the intramuscular and epimysial tissues prior to preparation of the labile fraction. These values are presented in Table 8.

#### A. Intramuscular Tissues

The nitrogen concentrations were found to be lowest in the younger fetal group, and to significantly increase during fetal and early post natal growth. There were no age or sex associated significant differences among the yearling and adult groups. The yearling and adult intramuscular nitrogen concentrations were similar, averaging 153.4 and 155.6 mg per g lyophilized-defatted tissue in Trials I and II, respectively.

Intramuscular collagen concentrations, in contrast to the nitrogen concentrations, were highest in the younger fetal group and decreased significantly during fetal and early post natal growth. There were no sex associated differences found between the young adult groups. The intramuscular collagen concentrations were similar in all adult groups, averaging 33.6 and 30.5 mg per g lyophilized-defatted tissue in Trials I and II, respectively. These results are consistent with the histochemical results reported earlier in this thesis.

Table 8. Bovine TB Intramuscular and Epimysial Nitrogen and Collagen Concentrations

| Age and Sex Groups | Number of Animals[2] | Concentration (mg/g tissue)[1] Nitrogen | | Collagen | |
|---|---|---|---|---|---|
| | | Mean | ±S.D. | Mean | ±S.D. |
| TRIAL I | | Intramuscular Tissue | | | |
| FI | 2 | 123.0[a] | 5.7 | 114.5[a] | 9.4 |
| FII | 3 | 131.3[b] | 3.5 | 89.1[b] | 5.7 |
| I | 3 | 153.3[c] | 2.1 | 29.1[c] | 0.5 |
| IIf | 4 | 153.8[c] | 4.1 | 36.2[c] | 6.0 |
| IIm | 4 | 155.5[c] | 3.3 | 32.6[c] | 1.3 |
| III | 4 | 150.8[c] | 1.3 | 32.0[c] | 6.3 |
| | | Epimysial Tissue | | | |
| I | 3 | 173.7[a] | 2.9 | 695.9[a] | 17.6 |
| IIf | 4 | 178.3[a] | 1.5 | 734.4[a] | 37.5 |
| IIm | 4 | 173.0[a] | 0.8 | 808.7[b] | 13.9 |
| III | 4 | 174.5[a] | 3.7 | 868.5[c] | 35.4 |
| TRIAL II | | Intramuscular Tissue | | | |
| IIf | 3 | 156.0[a] | 1.0 | 28.0[a] | 2.6 |
| IIm | 3 | 155.7[a] | 2.1 | 32.8[a] | 8.8 |
| III | 2 | 155.0[a] | 0.0 | 30.8[a] | 3.9 |
| | | Epimysial Tissue | | | |
| IIf | 3 | 175.0[a] | 2.0 | 762.3[a] | 26.9 |
| IIm | 3 | 176.7[a] | 2.1 | 758.3[a] | 40.5 |
| III | 2 | 174.5[a] | 0.7 | 823.8[b] | 4.5 |

1 Lyophilized-defatted basis.

2 Number of pooled samples in the case of fetal groups FI and FII

a,b,c - Means in the same vertical row, within a tissue, with different superscripts are significantly different ($P<0.05$).

B. Epimysial Tissues

There were no significant differences in the epimysial nitrogen concentrations among the age and sex groups. The epimysial nitrogen concentrations averaged 175.2 and 175.5 mg per g lyophilized-defatted tissue in Trials I and II, respectively. The epimysial collagen concentration was found to be significantly higher in the mature adult group than in the younger post natal groups in both Trials I and II. The collagen concentration of the young male adult group (IIm) was found to be significantly higher than that of the young female adult group (IIf) in Trial I. No significant difference was found between the male and female young adult groups in Trial II. However, in this trial, ages were not accurately known; consequently it is possible that the ages were considerably different and therefore overcame any sex differences that would be seen in groups of animals of similar ages.

C. Comparison of Intramuscular and Epimysial Tissues

On a lyophilized-defatted tissue basis, the nitrogen and collagen concentrations of the epimysial tissues were approximately 13% and 24 fold greater than those of the intramuscular tissues, respectively.

(ii) Discussion

The lower concentration of fetal intramuscular nitrogen is likely due to the relatively higher concentrations of ground substance and cell wall constituents as indicated by the higher concentrations of total hexosamine (see Table 13). These components contain relatively smaller amounts of nitrogen. The decreasing intramuscular collagen

concentration during fetal and post natal growth is undoubtedly related to the simultaneous increase of myofibrillar and sarcoplasmic protein concentrations associated with myofiber hypertrophy (Lawrie, 1966). The almost constant collagen concentration in the post natal groups is in agreement with reports by Hill (1966), Field et al. (1969) and Cross et al. (1973). Significantly higher epimysial collagen concentrations in the mature adult group than in the younger adult groups is probably due to the relatively lower proportion of ground substance in the former groups (see Table 13). This observation is supported by Kondo et al. (1971) who reported an increased hydroxyproline concentration with age in lyophilized-defatted chicken skin. Bailey (1968) suggested that there is an age associated increase in the collagen concentration in tissues such as skin, blood vessels and joints. Whereas Cormier et al. (1971), studying the bovine SM muscle epimysium, reported that, on a lyophilized-defatted basis, collagen concentrations were significantly higher in new born animals than in heifers (14.5 months) and old cows (10.5 years) with no significant difference between the latter two groups. The observations of these authors are not consistent with the literature. Cormier et al. (1971) state that their observations are consistent with those of Goll et al. (1963); however, these latter authors reported collagen concentrations for intramuscular tissue but not for epimysial tissue.

Although not significant in Trial II, the significantly higher epimysial collagen concentrations in young adult males than in young adult females, observed in Trial I, may be due to the selective action

of androgens. Similarly, the literature indicates a higher collagen concentration in males than in females in rat tendons (Kao and McGavack, 1959) and in the chicken aorta (Cembrano et al. 1960). Herrick and Brown (1952) studied sex hormone effects on collagen in chicken muscle and skin, and found a decreased collagen nitrogen concentration after caponization or diethylstilbestrol treatment and an increased collagen nitrogen concentration in capons treated with testosterone propionate. Possibly the higher collagen concentration of the epimysium from male animals reflects a need for a stronger connective tissue framework to support and to transfer force generated by a larger muscle mass. In this study, the TB muscle from the young adult male animals 1.6 years of age weighed 3623 g, while the TB muscle from the young adult female animals 2.0 years of age weighed only 1756 g.

The higher nitrogen concentrations in the epimysial tissues reflect the higher collagen concentrations, as collagen contains relatively more nitrogen than do most other tissue proteins (Eastoe and Courts, 1963).

2. Analysis of the Labile Fraction

(i) Results

A. Labile Collagen Concentrations

As shown in Figures 7 and 8, the percentage of the intramuscular and epimysial collagen that was solubilized by heating in a 0.15M NaCl solution at 77C for 1 h (labile collagen) decreased significantly with advancing age in both Trials I and II. The percentage of the collagen

FIGURE 7 Percentage Labile Collagen in Bovine TB Muscle (Trial I)

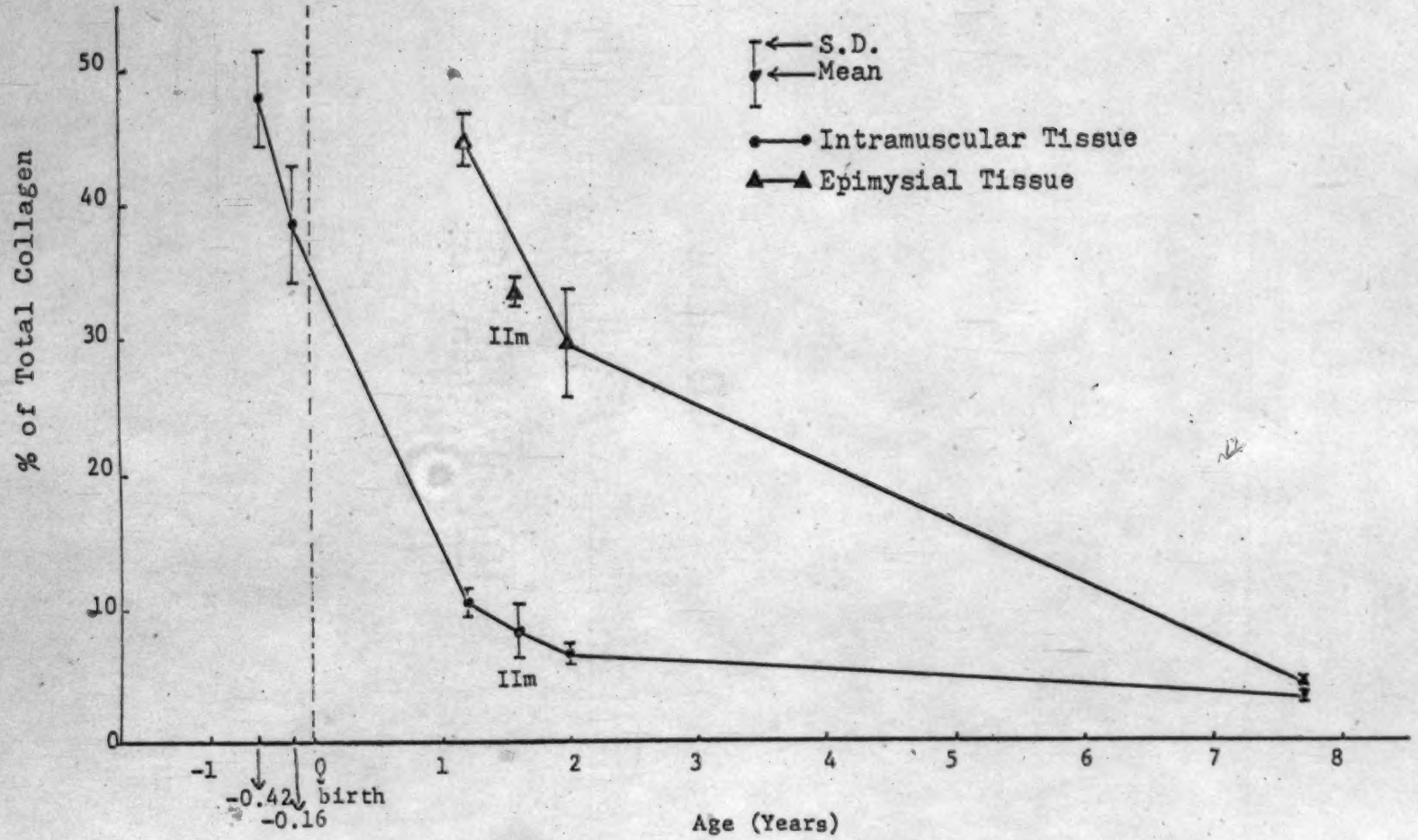
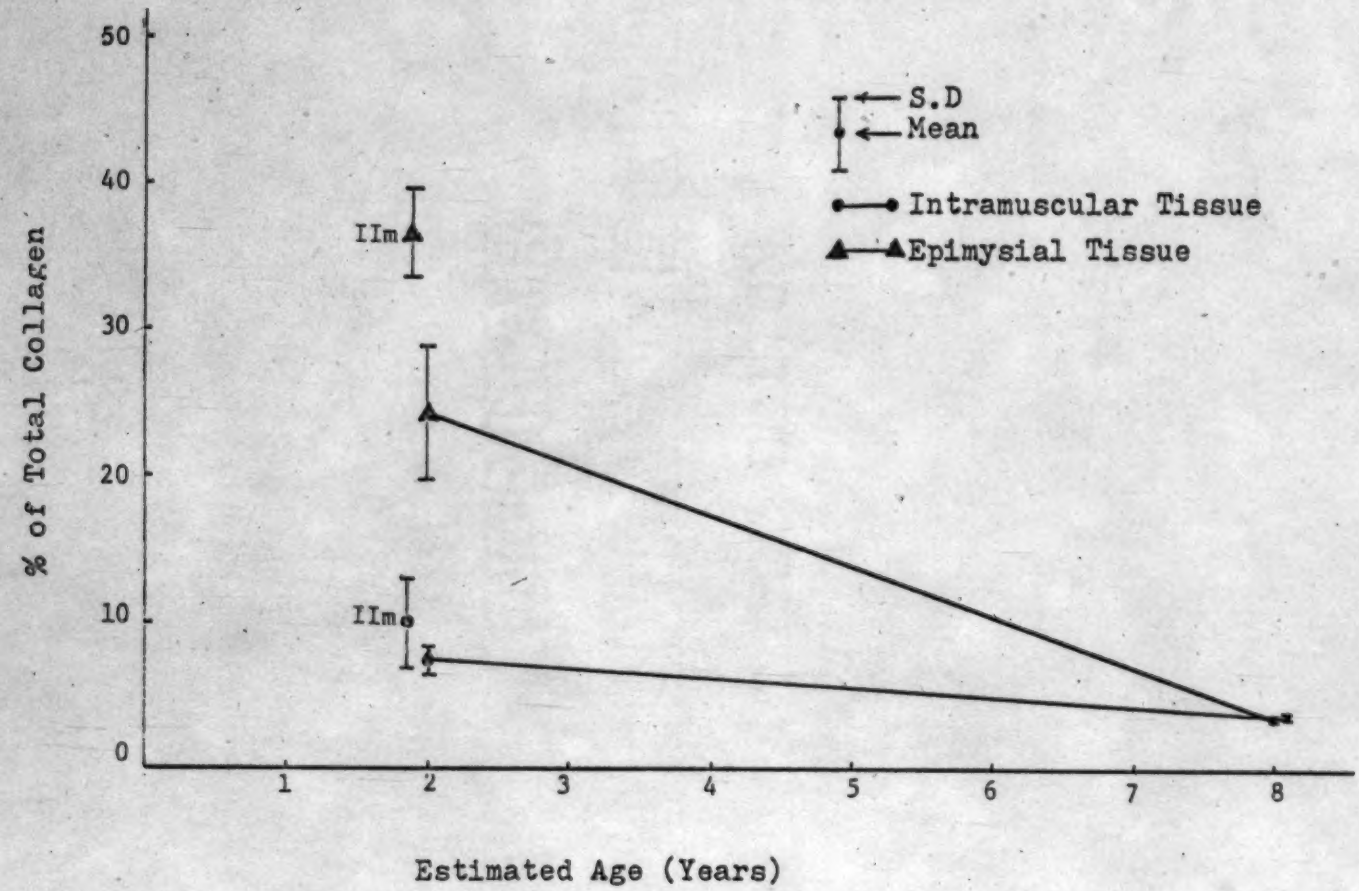


FIGURE 8 Percentage Labile Collagen in Bovine TB Muscle (Trial II)



that was labile in the epimysial tissue was approximately three to five times higher than that in the intramuscular tissue in both the yearling and young adult groups. In Trial I, 33.5% to 44.5% and in Trial II, 24.3% to 36.5% of the epimysial collagen was labile, while in Trial I only 6.7% to 11.6% and in Trial II only 7.5% to 10.0% of the intramuscular collagen was labile. Whereas, in the mature adult groups, the values were much lower yet similar in both tissues. In Trial I, 4.5% and in Trial II, 3.5% of the epimysial collagen was labile, and in Trial I, 3.5% and in Trial II, 3.7% of the intramuscular collagen was labile. The percentage labile collagen was significantly greater in the young female adults than in the young male adults in Trial II, but not in Trial I.

When expressed on a lyophilized-defatted basis, the intramuscular and epimysial concentrations of labile collagen were again shown to decrease with advancing animal age in both Trials I and II (Table 9). Fetal intramuscular labile collagen concentrations were 10 to 20 times higher than yearling or young adult intramuscular labile collagen concentrations, while in the mature adult groups, intramuscular labile collagen concentrations were about one-half to one-third lower than those of other post natal groups. These observations were significant at the 5% level. There were no significant differences in the intramuscular labile collagen concentrations between the yearling and young adult groups. Also, no significant sex related differences were found in the amounts of labile collagen in the intramuscular tissues.

The amount of epimysial labile collagen in the TB muscle from mature adults was six to ten times smaller than that found in the

1/10 to 1/16

Table 9. Composition of Bovine TB Labile Fraction

| Age and Sex Groups | Number of Animals | Collagen (mg/g)[1] | | Hexosamine (mg/g)[1] | | Sialic acid (mg/g)[1] | |
|---|---|---|---|---|---|---|---|
| | | Mean | ±S.D. | Mean | ±S.D. | Mean | ±S.D. |
| TRIAL I | | | | Intramuscular Tissue | | | |
| FI | 2 | $55.00^{a}$ | 0.42 | $4.11^{a}$ | 0.00 | $1.42^{a}$ | 0.41 |
| FII | 3 | $34.57^{b}$ | 6.00 | $3.03^{b}$ | 0.13 | $0.93^{b}$ | 0.01 |
| I | 3 | $3.03^{c}$ | 0.35 | $0.41^{c}$ | 0.08 | Trace[2] | - |
| IIf | 4 | $2.43^{c}$ | 0.19 | $0.41^{c}$ | 0.05 | Trace | - |
| IIm | 4 | $2.73^{c}$ | 0.69 | $0.36^{c}$ | 0.05 | Trace | - |
| III | 4 | $1.07^{d}$ | 0.10 | $0.39^{c}$ | 0.03 | Trace | - |
| | | | | Epimysial Tissue | | | |
| I | 3 | $311.74^{a}$ | 7.54 | $1.86^{a}$ | 0.12 | Trace | - |
| IIf | 4 | $218.00^{b}$ | 36.79 | $1.75^{a}$ | 0.19 | Trace | - |
| IIm | 4 | $269.17^{b}$ | 12.11 | $1.37^{b}$ | 0.14 | Trace | - |
| III | 4 | $39.30^{c}$ | 3.62 | $1.34^{b}$ | 0.17 | Trace | - |
| TRIAL II | | | | Intramuscular Tissue | | | |
| IIf | 3 | $2.13^{ab}$ | 0.42 | $0.43^{a}$ | 0.01 | Trace | - |
| IIm | 3 | $3.10^{a}$ | 0.35 | $0.41^{a}$ | 0.03 | Trace | - |
| III | 2 | $1.20^{b}$ | 0.14 | $0.38^{a}$ | 0.01 | Trace | - |
| | | | | Epimysial Tissue | | | |
| IIf | 3 | $184.80^{a}$ | 35.56 | $1.76^{a}$ | 0.31 | Trace | - |
| IIm | 3 | $281.33^{b}$ | 21.09 | $1.13^{b}$ | 0.18 | Trace | - |
| III | 2 | $28.60^{c}$ | 2.12 | $0.93^{b}$ | 0.17 | Trace | - |

[1] Lyophilized-defatted basis.

[2] <0.2 (mg/g).

a,b,c,d,- means in the same vertical row, within a tissue, with different superscripts are significantly different ($P < 0.05$).

yearlings and young adults. The epimysial tissue from young adult males contained more labile collagen than did similar tissue from young adult females in both Trials I and II. However, this difference was only significant in Trial II.

Labile collagen concentrations expressed on a lyophilized-defatted basis were approximately 30 to 100 times higher in epimysial tissues than in intramuscular tissues from animals of comparable ages.

B. Labile Hexosamine and Sialic Acid Concentrations

The concentrations of labile hexosamine and sialic acid in lyophilized-defatted tissue are presented in Table 9. The intramuscular labile hexosamine concentration decreased significantly during fetal growth. The intramuscular labile hexosamine concentrations in the fetal groups were approximately ten times greater than those in the yearling and adult groups. There were no significant differences found in the intramuscular labile hexosamine concentrations among the post natal groups in either Trials I or II.

The epimysial labile hexosamine concentration was significantly lower in the mature adult group than in the yearling and young female adult groups, and was significantly lower in young male adult groups than in young female adult groups in both Trials I and II. Labile hexosamine concentrations were approximately three to four times higher in epimysial tissues than in intramuscular tissues from animals of comparable ages.

The intramuscular labile sialic acid concentration was found to

decrease significantly during fetal growth. The labile fraction obtained from the younger fetal group contained 1.42 mg sialic acid per g lyophilized-defatted tissue, while the older fetal group contained 0.93 mg sialic acid per g lyophilized-defatted tissue. In the post natal animals, negligible amounts (<0.2 mg per g lyophilized-defatted tissue) of labile sialic acid were found in both the intramuscular and epimysial tissues.

C. Soluble Nonscleroprotein Nitrogen Concentrations

Soluble nonscleroprotein nitrogen is defined as the difference between soluble protein nitrogen and soluble collagen nitrogen. The SNSPN concentrations measured in this study are shown in Table 10. The intramuscular SNSPN concentration was found to be highest in the younger fetal group and to decrease significantly during fetal growth. Both fetal groups had a significantly higher intramuscular SNSPN concentration than did the post natal groups. A slight decrease in the intramuscular SNSPN concentration was observed during post natal growth. The epimysial SNSPN concentration was observed to be significantly lower in the mature adult group than in the remaining younger groups. The mature adult groups had a SNSPN concentration about one-half as high as did the younger groups. In both Trials I and II, intramuscular and epimysial tissues contained more or less similar concentrations of SNSPN in yearling and young adult groups, while in the mature adult groups, the epipmysial SNSPN concentrations were approximately one-half of the intramuscular SNSPN concentrations in both trials.

Table 10. Bovine TB Soluble Nonscleroprotein Nitrogen

| Age and Sex Groups | Soluble Nonscleroprotein Nitrogen | |
|---|---|---|
| | Mean | ±S.D. |
| **TRIAL I** | **Intramuscular Tissue** | |
| FI | 31.6[1,a] | 0.1 |
| FII | 26.7[b] | 1.6 |
| I | 19.6[c] | 1.6 |
| IIf | 19.1[cd] | 0.4 |
| IIm | 16.8[e] | 0.9 |
| III | 17.7[de] | 0.5 |
| | **Epimysial Tissue** | |
| I | 15.7[a] | 0.5 |
| IIf | 17.2[a] | 6.5 |
| IIm | 17.2[a] | 1.3 |
| III | 7.2[b] | 0.4 |
| **TRIAL II** | **Intramuscular Tissue** | |
| IIf | 17.8[a] | 0.5 |
| IIm | 18.9[a] | 0.3 |
| III | 18.1[a] | 0.4 |
| | **Epimysial Tissue** | |
| IIf | 17.9[a] | 5.8 |
| IIm | 20.4[a] | 2.8 |
| III | 9.8[b] | 2.3 |

[1] mg per g lyophilized-defatted tissue.

a,b,c,d,e - Means in the same vertical row, within a tissue, with different superscripts are significantly different ($P<0.05$).

(ii) Discussion

The decrease in the percentage labile collagen with advancing animal age (Figures 7 and 8) is in agreement with the observations of Goll et al. (1964b), Hill (1966), Herring et al. (1967), and Cross et al. (1973). This decrease is likely due to an increase in the extent of crosslinkage of muscle collagen with advancing animal age (Verzár, 1964). An increased degree of inter and intramolecular crosslinking will decrease the solubility of collagen. The rate of decrease of the percentage of labile collagen was found to be slower in epimysial tissues than in intramuscular tissues (Figures 7 and 8). If rate of collagen maturation can be measured as rate of decrease in solubility, epimysial collagen may be said not to "mature" as rapidly as does intramuscular collagen.

A higher epimysial labile collagen concentration was observed in males than in females. Similarly, Kao and McGavack (1959) studied tendons obtained from 3 to 5 week old rats, and reported that the amount of saline and alkali soluble protein is higher in males than in females.

Skeletal muscle labile hexosamine and sialic acid concentrations have not been previously reported in the literature. However, Houck et al. (1961) studied salt soluble hexosamine in the rat skin, and reported that no insoluble hexosamine was found until the animals reached a weight of 270 g. The only report in the literature dealing with skeletal muscle sialic acid has been the recent report of Vandenburgh et al. (1974) who demonstrated the presence of sialic acid in the rat sarcolemma.

Analysis of SNSPN has not been previously reported for skeletal muscle but has been reported for other tissues such as skin and tendon (Kao and McGavack, 1959). Lower SNSPN concentrations with increasing age were obtained in this study. Similarly, Kao and McGavack (1959) reported that nonscleroprotein concentrations decreased with increasing age in tendon and skin from 3 week to 2 year old rats.

## III. BOVINE TB MUSCLE GAG AND OTHER HEXOSAMINE CONTAINING SUBSTANCES

Throughout Section III of this thesis, only tissues from Trial I animals were studied, since the history of animals in Trial II was not adequately known.

### 1. Results

#### (i) Analysis I: Quantitative Analysis of Bovine Muscular GAG

Total GAG concentrations in lyophilized-defatted TB muscle were measured as uronic acid in the 0.4M, 1.2M and 2.1M NaCl fractions obtained by the CPC method (Table 11). The percentage of each of the isomeric galactosaminoglycans, Ch4-S, Ch6-S and DS in the 1.2M NaCl fraction was determined enzymatically and is presented in Table 12.

In both the 0.4M and 1.2M NaCl fractions of intramuscular tissue, the concentration of uronic acid decreased significantly during fetal growth. Both fetal groups had significantly higher concentrations of uronic acid in these fractions than did the post natal groups. The younger fetal group had a significantly higher uronic acid concentration in the 2.1M NaCl fraction than did the older fetal group

Table 11. Uronic Acid Concentration of the Three GAG Fractions Obtained by the CPC Method.

| Age and Sex Groups | Number of Animals | Fractions | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.4M NaCl | | 1.2M NaCl | | 2.1M NaCl | | Total | |
| | | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. |
| | | | | | Intramuscular Tissue | | | | |
| FI | 2 | 552[a1] | 34 | 754[a] | 46 | 47[a] | 9 | 1353[a] | 83 |
| FII | 3 | 360[b] | 22 | 314[b] | 38 | 22[b] | 1 | 696[b] | 17 |
| I | 3 | 139[c] | 28 | 101[c] | 14 | 15[b] | 8 | 249[c] | 10 |
| IIf | 4 | 173[c] | 9 | 96[c] | 17 | 19[b] | 8 | 288[c] | 28 |
| IIm | 4 | 117[c] | 16 | 85[c] | 9 | 12[b] | 7 | 214[c] | 14 |
| III | 4 | 140[c] | 22 | 87[c] | 21 | 19[b] | 3 | 246[c] | 41 |
| | | | | | Epimysial Tissue | | | | |
| I | 3 | 643[a] | 54 | 1068[a] | 30 | 24[a] | 4 | 1735[a] | 80 |
| IIf | 4 | 731[a] | 114 | 894[a] | 102 | 24[a] | 13 | 1649[b] | 162 |
| IIm | 4 | 536[a] | 98 | 878[a] | 81 | 23[a] | 12 | 1438[bc] | 179 |
| III | 4 | 565[a] | 83 | 692[b] | 77 | 19[a] | 10 | 1276[c] | 121 |

[1] µg uronic acid per g lyophilized-defatted tissue.

a, b, c, - Means in the same vertical row, within a tissue, with different superscripts are significantly different ($P < 0.05$).

Table 12. Percentage of Each Isomeric Galactosaminoglycan and the Uronic Acid Color Yield Ratio in the 1.2M NaCl Fraction.

| Age and Sex Groups | Number of Animals | Percentage of each Galactosaminoglycan | | | | | | Uronic Acid Color Yield Ratio[1] | |
|---|---|---|---|---|---|---|---|---|---|
| | | Ch4-S | | DS | | Ch6-S | | | |
| | | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. |
| | | Intramuscular Tissue | | | | | | | |
| FI | 2 | 13.6[a] | 3.3 | 57.8[a] | 8.1 | 28.8[a] | 4.8 | 0.96[a] | 0.01 |
| FII | 3 | 10.3[a] | 2.6 | 72.2[b] | 0.6 | 17.6[b] | 3.2 | 0.88[b] | 0.07 |
| I | 3 | 12.9[a] | 2.7 | 72.6[b] | 4.6 | 14.5[b] | 1.9 | 0.85[bc] | 0.02 |
| IIf | 4 | 7.5[a] | 2.3 | 74.3[b] | 3.2 | 18.3[b] | 3.0 | 0.83[bc] | 0.03 |
| IIm | 4 | 9.9[a] | 2.9 | 75.1[b] | 5.5 | 15.1[b] | 2.7 | 0.84[bc] | 0.01 |
| III | 4 | 6.6[a] | 5.8 | 76.4[b] | 5.6 | 16.6[b] | 0.9 | 0.81[c] | 0.02 |
| | | Epimysial Tissue | | | | | | | |
| I | 3 | 2.3[a] | 0.7 | 84.5[a] | 2.3 | 13.5[a] | 2.3 | 0.74[a] | 0.03 |
| IIf | 4 | 3.1[a] | 1.8 | 80.8[a] | 8.4 | 16.1[a] | 7.9 | 0.73[a] | 0.01 |
| IIm | 4 | 1.7[a] | 0.4 | 81.8[a] | 2.7 | 16.6[a] | 3.0 | 0.75[a] | 0.02 |
| III | 4 | 1.5[a] | 0.3 | 84.3[a] | 2.3 | 14.1[a] | 2.4 | 0.72[a] | 0.03 |

[1]Dische:Bitter and Muir.

a, b, c, - Means in the same vertical row, within a tissue, with different superscripts are significantly different. ($P < 0.05$).

or the post natal groups. There were no significant differences in the uronic acid values among the post natal age and sex groups within each of the salt fractions.

Total intramuscular GAG concentration decreased significantly until the animals became yearlings with average uronic acid concentrations of 1353, 696 and 249 µg per g lyophilized-defatted tissue for the younger fetal, older fetal and yearling groups respectively. There were no significant differences in total intramuscular GAG concentrations (214 to 288 µg per g lyophilized-defatted tissue) in the TB muscles obtained from the post natal age and sex groups.

In the epimysial 1.2M NaCl fraction, the concentration of uronic acid continually decreased with increasing animal age with the mature adult group displaying a significantly lower concentration than the other post natal groups. No significant differences between sex groups were observed. In the epimysial 0.4M and 2.1M NaCl fractions, there were no significant differences observed in the uronic acid concentrations among the age and sex groups. The concentrations of total epimysial GAG were found to gradually and significantly decrease with advancing age from 1735 to 1276 µg per g lyophilized-defatted tissue. No significant difference was observed in the concentrations of total epimysial GAG between the sex groups.

A comparison of the average post natal intramuscular and epimysial uronic acid concentrations found in each salt fraction from the CPC method reveals differences in the GAG concentrations of these two

tissues. In the intramuscular samples, the 0.4M NaCl fraction contained the largest amount of uronic acid, or 57% of the total, while the 1.2M and 2.1M NaCl fractions contained 37% and 6% of the total uronic acid, respectively. In the epimysial tissues, the 1.2M NaCl fraction contained the largest amount of uronic acid, or 58% of the total, while the 0.4M and 2.1M NaCl fractions contained 41% and 2% of the total uronic acid, respectively. The intramuscular uronic acid fractionation patterns in the younger fetal samples tended to reflect that seen in the post natal epimysial tissues. An overall comparison of the post natal groups revealed that the epimysial tissues contained a six fold greater concentration of total uronic acid than did the intramuscular tissues.

Since the color yield of the carbazole reaction is affected by the structure of the GAG, a quantitative comparison is meaningful only when based on one of the following assumptions: each GAG fraction from the experimental groups contains only one type of GAG, or if there are two or more types present in a given fraction, their proportion is identical among all experimental groups. In order to confirm the observations in Analysis I, Analysis II was conducted, and each GAG fraction was measured as uronic acid and as hexosamine which produces an equivalent color yield for each GAG by the Elson-Morgan reaction.

Enzymatic analysis of the intramuscular 1.2M NaCl fraction indicated that the relative amounts of the isomeric galactosaminoglycans, Ch4-S, Ch6-S and DS in the younger fetal group differs significantly from the remaining older age groups. The younger

fetal groups were found to contain a significantly smaller amount of DS, averaging 57.8% versus 74.1% for the older fetal group and the post natal groups. They also contained a significantly larger amount of Ch6-S, averaging 28.8%, versus 16.4% for intramuscular tissues from the other experimental groups. These differences resulted in a significant decrease in the uronic acid color yield ratio during fetal growth (Table 12). The relative amounts of Ch4-S, DS and Ch6-S did not differ significantly among the older fetal and post natal groups. The average relative amounts of Ch4-S, DS and Ch6-S in intramuscular tissues were 9.4%, 74.1% and 16.4%, while in epimysial tissue, they averaged 2.1%, 82.8% and 15.0% respectively. These data indicate that the isomeric galactosaminoglycan patterns in these groups differ between intramuscular and epimysial tissues with a slightly lower percentage of DS and considerably higher percentages of Ch4-S in the intramuscular tissue. The uronic acid color yield ratio did not differ significantly among the older fetal and post natal groups with average values of 0.84 and 0.74 in the intramuscular and epimysial tissues respectively. The relatively lower value in epimysial tissue reflects the relatively higher concentration of DS.

(ii) Analysis II: Quantitative and Qualitative Analysis of Bovine TB Muscle GAG and Other Hexosamine Containing Substances

A. Uronic Acid Test of Non-GAG Fractions

All glycopeptide fractions and celite residues were tested for the presence of GAG as uronic acid. Five hundred to 1000 µg samples of the glycopeptide fraction, determined as hexosamine, were chromatographed

by means of Silica Gel-G thin layer chromatography. As shown in Figure 9, faint bands for uronic acid were obtained with all intramuscular and epimysial glycopeptide fractions. These bands were found to be smaller than those of a co-chromatographed 5 µg glucuronolactone sample. Therefore it is probable that less than 1% of the hexosamine present in the glycopeptide fraction was present in the form of GAG.

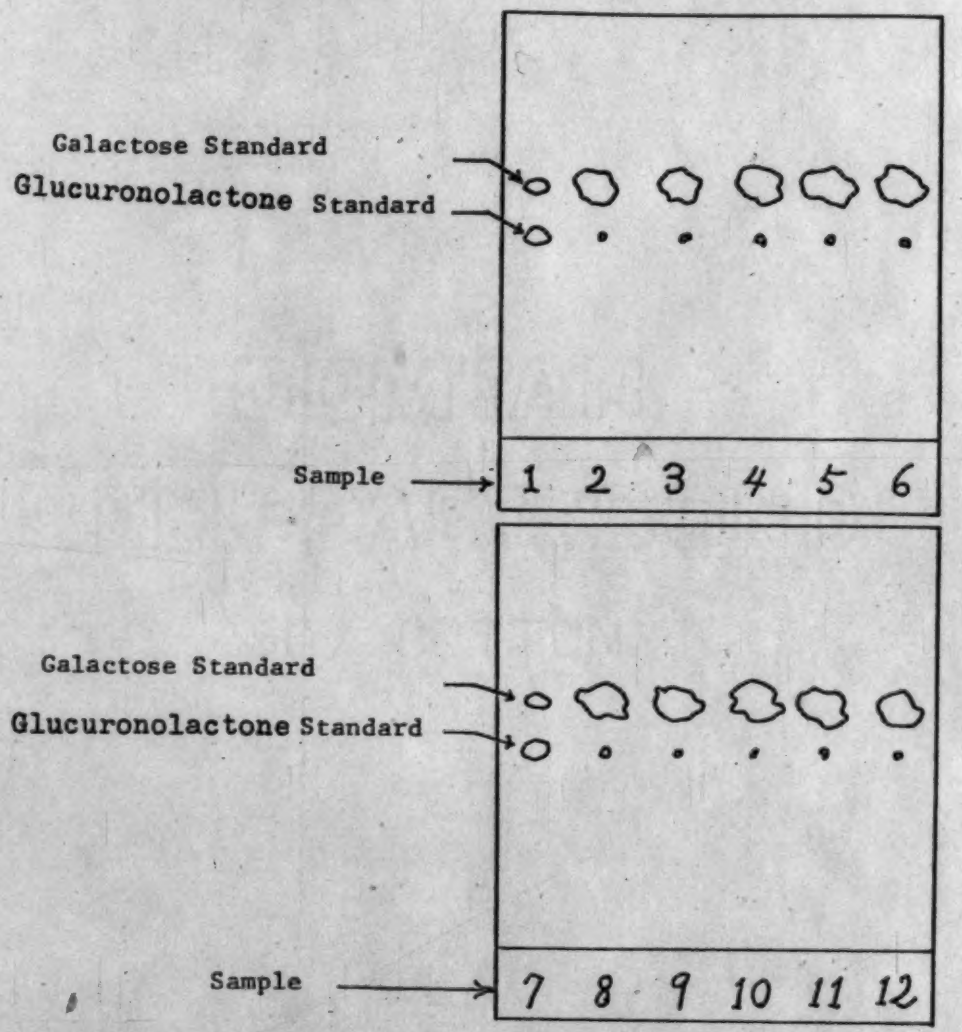
The presence of uronic acid in the celite residues was checked by means of the carbazole reaction (Bitter and Muir, 1962) directly in the glass centrifuge tubes. There were negligible amounts of GAG, as uronic acid, in this residue, accounting for 0.1 to 0.3% of the total uronic acid.

Consequently, it was considered that the loss of muscular GAG during the CPC method was negligible.

B. Percentage Hexosamine Released During Isolation and Fractionation of GAG by the CPC Method

The percentage hexosamine released during isolation and fractionation of GAG by the CPC method was expressed as a percentage of the total hexosamine released during proteolytic enzyme digestion plus the total hexosamine remaining in the undigested residue (i.e. total hexosamine). The percentages of hexosamine released from tissue by proteolytic digestion were 97.4% to 98.6% from fetal intramuscular tissue, 91.3% to 96.0% from post natal intramuscular tissue, and 96.3% to 97.3% from epimysial tissue (Table 13).

FIGURE 9 Uronic Acid Test of the Glycopeptide Fraction by Thin Layer Chromatography.




Sample: 1 and 7, standard galactose and glucuronolactone mixtures, 2, FI, 3, FII, 4, I, 5, IIf, 6, IIm, 8, III (2-6 and 8, intramuscular tissue ), 9, I, 10, IIf, 11, IIm, 12, III (9-12, epimysial tissue).

The intramuscular glycopeptide fraction contained a higher proportion of the total hexosamine (65.2% to 71.7%) than did the epimysial glycopeptide fraction (40.2% to 45.3%) of the post natal animals (Table 14).

The proportion of the total hexosamine present in the GAG fractions was found to be 21.1% to 24.8% in the fetal intramuscular tissues, 19.2% to 23.5% in post natal intramuscular tissues and 45.3% to 56.7% in post natal epimysial tissues (Table 14).

C. Hexosamine Recovery After Fractionation by the CPC Method

The total hexosamine recovered after fractionation of the digestion filtrate (Figure 4) was calculated by expressing the total amount of hexosamine present in the glycopeptide, 0.4M NaCl, 1.2M NaCl and 2.1M NaCl fractions (Table 14) as a percentage of the total amount present in the effluent (Table 13).

The percentage recoveries as shown in Table 14 were: 97.2% to 99.3% from fetal intramuscular tissue, 94.0% to 98.1% from post natal intramuscular tissue and 90.5% to 100.1% from epimysial tissue.

The majority of the bovine TB muscle GAG hexosamine recovered by the CPC method was found in the 0.4M and 1.2M NaCl fractions. The proportion of GAG hexosamine in the 2.1M NaCl fraction, calculated as a percentage of the total tissue GAG (Table 14) was 3% in fetal intramuscular tissue, 7% to 9% in post natal intramuscular tissue and 2% to 3% in epimysial tissue.

Table 13. Hexosamine Released During Proteolytic Digestion

| Age and Sex Groups | FI | FII | I | IIf | IIm | III |
|---|---|---|---|---|---|---|
| | | | Intramuscular Tissue | | | |
| (Fraction) Residue | 113[1] (2.6)[2] | 36 (1.4) | 50 (4.0) | 104 (8.7) | 68 (7.2) | 87 (7.8) |
| Filtrate | 4242 (97.4) | 2663 (98.6) | 988 (96.0) | 972 (91.3) | 874 (92.8) | 1028 (92.2) |
| Total Hexosamine | 4355 | 2699 | 1038 | 1076 | 942 | 1115 |
| | | | Epimysial Tissue | | | |
| (Fraction) Residue | - | - | 103 (3.7) | 79 (2.7) | 68 (3.2) | 80 (3.4) |
| Filtrate | - | - | 2677 (96.3) | 2766 (97.3) | 2053 (96.8) | 2249 (96.6) |
| Total Hexosamine | | | 2780 | 2845 | 2121 | 2329 |

[1] µg per g lyophilized-defatted tissue.

[2] % of the total hexosamine.

Table 14. Hexosamine Released During Fractionation by the CPC Method.

| Age and Sex Groups | FI | FII | I | IIf | IIm | III |
|---|---|---|---|---|---|---|
| (Fraction) | | | Intramuscular Tissue | | | |
| Glycopeptide | 3135[1] (72.0)[2] | 2020 (74.8) | 744 (71.7) | 702 (65.2) | 640 (67.9) | 762 (68.3) |
| 0.4M NaCl | 514 (11.8) | 324 (12.0) | 118 (11.3) | 132 (12.3) | 105 (11.1) | 115 (10.3) |
| 1.2M NaCl | 531 (T2.2) | 225 (8.3) | 85 (8.2) | 97 (9.0) | 85 (9.0) | 81 (7.3) |
| 2.1M NaCl | 35 (0.8) | 22 (0.8) | 20 (1.9) | 23 (2.2) | 15 (1.6) | 18 (1.6) |
| Total GAG | 1080 (24.8) | 571 (21.1) | 223 (21.5) | 252 (23.5) | 205 (21.7) | 214 (19.2) |
| 0.4M NaCl / 1.2M NaCl | 0.97 | 1.44 | 1.51 | 1.51 | 1.42 | 1.42 |
| Hexosamine Recovery (%) | 99.3 | 97.2 | 97.9 | 98.1 | 96.7 | 94.0 |
| (Fraction) | | | Epimysial Tissue | | | |
| Glycopeptide | - | - | 1251[1] (45.0)[2] | 1197 (42.1) | 853 (40.2) | 1054 (45.3) |
| 0.4M NaCl | - | - | 435 (15.6) | 407 (14.3) | 377 (17.8) | 445 (19.1) |
| 1.2M NaCl | - | - | 881 (31.7) | 859 (30.2) | 802 (37.8) | 590 (25.3) |
| 2.1M NaCl | - | - | 25 ((0.8) | 41 (1.4) | 24 (1.1) | 22 (0.9) |
| Total GAG | - | - | 1341 (48.1) | 1307 (45.9) | 1203 (56.7) | 1057 (45.3) |
| 0.4M NaCl / 1.2M NaCl | - | - | 0.49 | 0.47 | 0.47 | 0.75 |
| Hexosamine Recovery (%) | - | - | 96.8 | 90.5 | 100.1 | 93.9 |

[1] mg hexosamine released in each fraction per lyophilized-defatted tissue.

[2] % of the total hexosamine.

D. Concentration of Total Tissue GAG Hexosamine

The concentration of total GAG hexosamine was estimated to range from 571 to 1080 µg per g in the fetal intramuscular tissue, 205 to 252 µg per g in the post natal intramuscular tissue, and 1057 to 1341 µg per g in the epimysial tissue. All values are expressed on a lyophilized-defatted basis (Table 14). The post natal intramuscular concentrations are one sixth to one fifth of the epimysial concentrations.

E. Relative Proportions GAG in the 0.4M and 1.2M NaCl Fractions (0.4M NaCl: 1.2M NaCl Fraction)

A comparison of the relative amounts of GAG in the 0.4M and 1.2M NaCl fractions is presented in Table 14 as a ratio of the total hexosamine concentrations in the 0.4M and 1.2M NaCl fractions. This ratio was found to be 2 to 3 times higher in the intramuscular tissue, with values ranging from 1.42 to 1.51, than in the epimysial tissue, with values ranging from 0.47 to 0.75. The exception, however, was found in the younger fetal group, in which a ratio value of 0.97 implies nearly equal proportions of each of the two NaCl fractions. Similar values for the relative amounts of GAG in the 0.4M NaCl and 1.2M NaCl fractions can also be calculated from uronic acid analysis of these same fractions in Analysis I (Table 11). The values for the 0.4M NaCl : 1.2M NaCl ratio for intramuscular tissue range from 1.15 to 1.80, while values for epimysial tissue range from 0.60 to 0.82. The value for the younger fetal groups was 0.73.

## F. Effect of Age and Sex on Bovine TB Muscle GAG, Glycopeptide, and Sialic Acid Concentrations and Ratio of Total GAG to Collagen

### (a) Effect of Age

A comparison of intramuscular and epimysial GAG hexosamine (Table 14) and uronic acid (Table 15) concentrations obtained in Analysis II with uronic acid concentrations (Table 11) obtained in Analysis I revealed similar age and sex related patterns.

Fetal growth was associated with a rapid decrease in the intramuscular total GAG concentration. Total GAG concentrations were; 1080 µg/g as hexosamine and 1340 µg/g as uronic acid in the younger fetal group and 571 µg/g as hexosamine and 716 µg/g as uronic acid in the older fetal group. There was little change amoung the post natal groups, with GAG concentrations ranging from 205 to 252 µg/g as hexosamine and 212 to 255 µg/g as uronic acid. The GAG concentration of each salt fraction decreased during fetal growth, and remained relatively constant thereafter.

The total GAG concentration in mature adult epimysium was approximately 21% lower than that in the yearling epimysium, 1057 µg/g as hexosamine and 1216 µg/g as uronic acid versus 1341 µg/g as hexosamine and 1552 µg/g as uronic acid. There appeared to be an overall decrease in GAG concentration related to increasing animal age. The GAG concentration in the 1.2M NaCl epimysial fraction was considerably lower in mature adults than in the remaining younger groups, while that in the 0.4M and 2.1M NaCl fractions did not differ greatly.

The intramuscular glycopeptide hexosamine concentration decreased during fetal growth from 3135 to 2020 µg/g. The concentration in the

Table 15. Uronic Acid Concentrations in the GAG Fractions Obtained by the CPC Method.

| (Age and Sex Groups) (Fraction) | FI | FII | I | IIf | IIm | III |
|---|---|---|---|---|---|---|
| | | | Intramuscular Tissue | | | |
| 0.4M NaCl | 552[1] | 357 | 123 | 140 | 109 | 123 |
| 1.2M NaCl | 755 | 335 | 95 | 99 | 95 | 90 |
| 2.1M NaCl | 33 | 24 | 10 | 16 | 8 | 14 |
| Total | 1340 | 716 | 228 | 255 | 212 | 227 |
| | | | Epimysial Tissue | | | |
| 0.4M NaCl | - | - | 466 | 422 | 412 | 503 |
| 1.2M NaCl | - | - | 1056 | 1111 | 1005 | 687 |
| 2.1M NaCl | - | - | 30 | 38 | 19 | 26 |
| Total | | | 1552 | 1571 | 1436 | 1216 |

[1] µg uronic acid per g lyophilized-defatted tissue.

post natal groups remained relatively constant, ranging from 640 to 762 µg per g lyophilized-defatted tissue (Table 14). The epimysial glycopeptide hexosamine concentration was found to decrease gradually with increasing animal age. The value of mature adults (1054 µg per g) was approximately 16% lower than that of yearlings (1251 µg per g) on a lyophilized-defatted basis.

The sialic acid concentration was determined in the proteolytic enzyme digested effluent (Table 16). The intramuscular concentration of sialic acid was found to be highest in the younger fetal group and to decrease during fetal growth, 842 µg per g versus 521 µg per g, while the concentration in the post natal age groups remained relatively constant, ranging from 184 to 193 µg per g lyophilized-defatted tissue. The epimysial concentration of sialic acid was found to be somewhat higher in the yearlings, 344 µg per g, and young female adults, 328 µg per g, than in the young male adults, 276 µg per g. The epimysium from the mature adult group contained the lowest concentrations of sialic acid, 250 µg per g lyophilized-defatted tissue. A general decrease in epimysial sialic acid concentration was associated with increasing animal age.

The total GAG to collagen ratio is often used as an estimate of the relative quantity of ground substance to collagenous tissue (Sobel, 1967). In this study, each total GAG value measured either as hexosamine (Table 14) or as uronic acid (Table 11), was divided by the corresponding hydroxyproline value.

Table 16. Sialic Acid Concentrations in the Proteolytic Enzyme Digestion Filtrate.

| Age and Sex Groups | Intramuscular Tissue | Epimysial Tissue |
|---|---|---|
| FI | 842[1] | - |
| FII | 521 | - |
| I | 186 | 344 |
| IIf | 193 | 328 |
| IIm | 184 | 276 |
| III | 191 | 250 |

[1] µg sialic acid per g lyophilized-defatted tissue.

Hydroxyproline values were obtained by dividing the collagen values in Table 9 by 7.25. Each of the ratios obtained was plotted against age as shown in Figures 10 and 11.

The intramuscular hexosamine : hydroxyproline ratio decreased rapidly during fetal growth. Thereafter the decrease was found to be slow. Statistical analysis for the ratio based on the uronic acid concentration (Figure 11) showed no significant change among the post natal female groups. This observation apparently reflects the small changes in the total intramuscular GAG fraction and collagen concentrations during this period. The epimysial hexosamine : Hydroxyproline ratio constantly decreased as animals grew older. The greatest decrease was found between young female adult and mature adult groups. Statistical analysis for the ratio based on the uronic acid concentration (Figure 11) showed the difference between yearlings and mature adults, and young female adults and mature adults to be significant.

(b) Effect of Sex

The effect of sex on total tissue GAG, glycopeptide, sialic acid and the ratio of total GAG to collagen was estimated by comparing the young female adult animals averaging 2.0 years of age with the young male adult animals averaging 1.6 years of age.

Both intramuscular and epimysial tissues from the male group were observed to have somewhat lower total GAG determined as uronic acid

FIGURE 10 Total GAG Hexosamine to Hydroxyproline Ratio

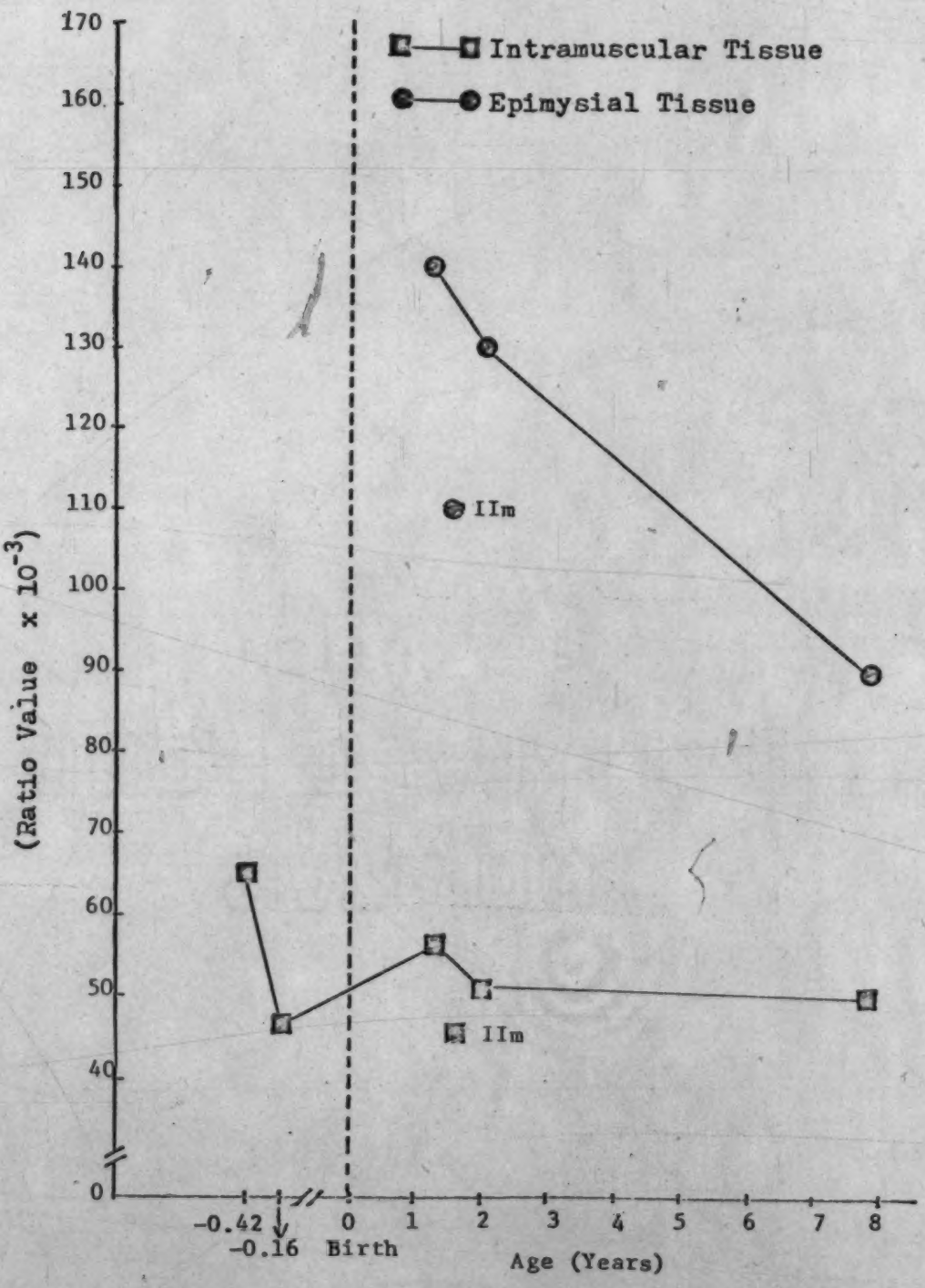
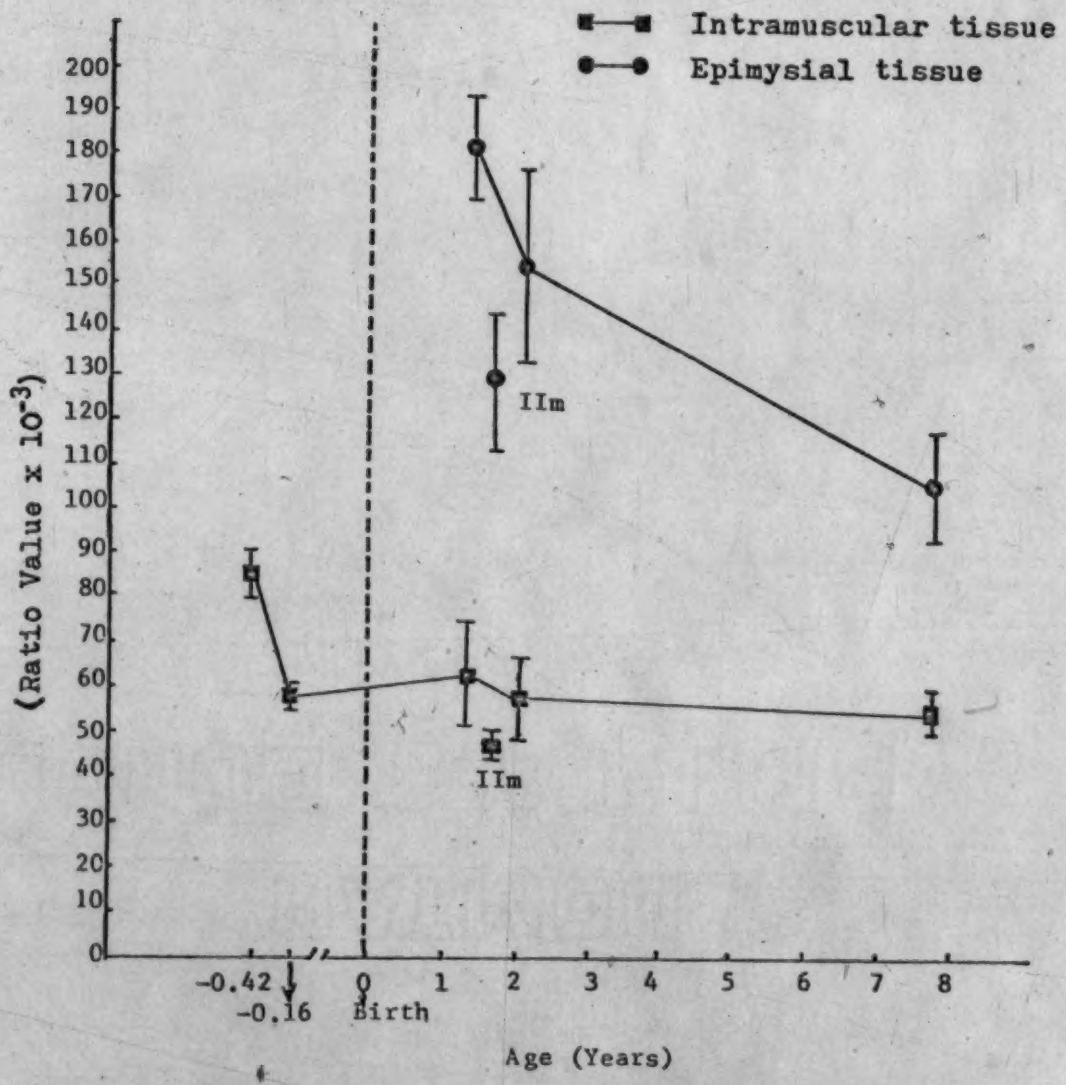


FIGURE 11 Total GAG Uronic Acid to Hydroxyproline Ratio



(Tables 11 and 15), total GAG determined as hexosamine (Table 14), glycopeptide hexosamine (Table 14) and sialic acid (Table 16) concentrations. Both the 0.4M and 1.2M NaCl GAG fractions were also found to be lower in the male than in the female intramuscular and epimysial tissues. Both the intramuscular and epimysial hexosamine : hydroxyproline ratios were found to be lower in the young adult males than in the young adult females (Figure 10). Statistical analysis for the ratios based on the uronic acid concentration showed a significantly lower ratio for epimysial tissue in males than in females of a similar age (Figure 11).

G. Characterization of Each GAG Fraction by Chemical Analysis and Electrophoresis

Results of detailed chemical analysis of the two major GAG fractions (0.4M and 1.2M NaCl fractions) are presented in Table 17. The analytical data for the reference GAG are shown in Table 18. The 2.1M NaCl fraction was not subjected to the detailed chemical analyses due to the small amount of GAG present but was subjected to electrophoresis along with the 0.4M and 1.2M NaCl fractions. Electrophoretic observations are shown in Figures 12 and 13.

(a) 0.4M NaCl Fraction

A detailed chemical and electrophoretic analysis of the 0.4M NaCl GAG fraction indicated that this fraction was almost entirely composed of HA. The molar ratio of sulfate to hexosamine was very low (0.02 to 0.08). The uronic acid color yield ratio (Dische: Bitter and Muir) was 0.98 to 1.02. The molar ratio of uronic acid to hexosamine

Table 17. Chemical Analysis of the 0.4M and 1.2M NaCl Fractions.

Intramuscular Tissue

| Group | FI | | FII | | I | | IIf | | IIm | | III | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M NaCl Fraction | 0.4 | 1.2 | 0.4 | 1.2 | 0.4 | 1.2 | 0.4 | 1.2 | 0.4 | 1.2 | 0.4 | 1.2 |
| Uronic Acid Color Yield Ratio: | | | | | | | | | | | | |
| Dische Bitter and Muir | 1.00 | 0.90 | 1.00 | 0.86 | 1.00 | 0.87 | 0.98 | 0.84 | 1.01 | 0.84 | 0.98 | 0.82 |
| Dische Orcinol | - | 1.45 | - | 1.32 | - | 1.30 | - | 1.25 | - | 1.28 | - | 1.28 |
| Uronic acid[1] / Hexosamine | 1.08 | 1.42 | 1.12 | 1.49 | 1.04 | 1.12 | 1.06 | 1.02 | 1.04 | 1.12 | 1.07 | 1.10 |
| Glucosamine[1] / Galactosamine | 25 | 0.06 | 30 | 0.05 | 73 | 0.07 | 87 | 0.04 | 86 | 0.10 | 51 | 0.05 |
| $SO_4$[1] / Hexosamine | 0.02 | 0.90 | 0.03 | 1.01 | 0.05 | 1.12 | 0.08 | 0.95 | 0.04 | 1.07 | 0.04 | 0.95 |
| Hyaluronidase susceptibility[2] | 99 | 58 | 97 | 50 | 97 | 47 | 97 | 41 | 98 | 46 | 98 | 43 |

Table 17. (continued)

| Group | Epimysial Tissue I | | IIf | | IIm | | III | |
|---|---|---|---|---|---|---|---|---|
| M NaCl Fraction | 0.4 | 1.2 | 0.4 | 1.2 | 0.4 | 1.2 | 0.4 | 1.2 |
| Uronic Acid Color Yield Ratio: | | | | | | | | |
| Dishce / Bitter and Muir | 0.99 | 0.74 | 0.98 | 0.73 | 0.99 | 0.72 | 1.02 | 0.72 |
| Dische / Orcinol | - | 1.07 | - | 1.03 | - | 1.09 | - | 1.05 |
| Uronic acid[1] / Hexosamine | 1.04 | 1.18 | 1.04 | 1.29 | 1.09 | 1.26 | 1.13 | 1.16 |
| Glucosamine[1] / Galactosamine | 80 | 0.05 | 47 | 0.05 | 40 | 0.05 | 104 | 0.04 |
| $SO_4$[1] / Hexosamine | 0.03 | 1.02 | 0.02 | 1.11 | 0.03 | 1.02 | 0.02 | 1.20 |
| Hyaluronidase susceptibility[2] | 98 | 30 | 96 | 29 | 96 | 37 | 99 | 28 |

[1]Molar ratio.

[2]% fall of turbidity.

Table 18. Chemical Analysis of Reference GAG

| Reference GAG[1] | HA | Ch4-S | DS | Ch6-S | HS | HP | KS |
|---|---|---|---|---|---|---|---|
| Uronic Acid Color Yield Ratio: | | | | | | | |
| $\frac{\text{Dische}}{\text{Bitter and Muir}}$ | 1.00 | 0.99 | 0.62 | 1.00 | 1.16 | 1.19 | - |
| $\frac{\text{Dische}}{\text{Orcinol}}$ | 1.58 | 1.70 | 0.45 | 1.73 | 2.63 | 4.00 | - |
| $\frac{SO_4^{2}}{\text{Hexosamine}}$ | 0.00 | 0.97 | 1.29 | 0.98 | 0.99 | 2.33 | 1.17 |
| $\frac{\text{Glucosamine}^{2}}{\text{Galactosamine}}$ | 1000 | 0.007 | 0.029 | 0.001 | 500 | 1000 | 71 |
| Hyaluronidase susceptibility[3] | 98.0 | 95.4 | 19.2[4] | 98.6 | 1.1 | 4.5 | 5.0 |

[1] Gifts from Dr. Mathews except the DS for the uronic acid data.

[2] Molar ratio from standard GAG analytical data sheet (Mathews, personal communication).

[3] % fall of turbidity.

[4] The 19.2% fall in turbidity in reference DS is expected because of the D-glucuronic acid content of the preparation (Mathews, personal communication).

was 1.05 to 1.16. The constituent hexosamine was almost entirely glucosamine. Testicular hyaluronidase susceptibility of this fraction was 96 to 99%. Electrophoresis in 0.1M phosphate buffer and subsequent staining of the 0.4M NaCl fraction revealed a single discrete band for all intramuscular and epimysial tissues studied (Figure 12a). This band had a Rf value identical to the HA standard.

The proportion of HA in the total GAG pool was found to be 48% to 57% in intramuscular tissue and 31% to 42% in epimysial tissue, based on the hexosamine concentration of each GAG fraction (Table 14).

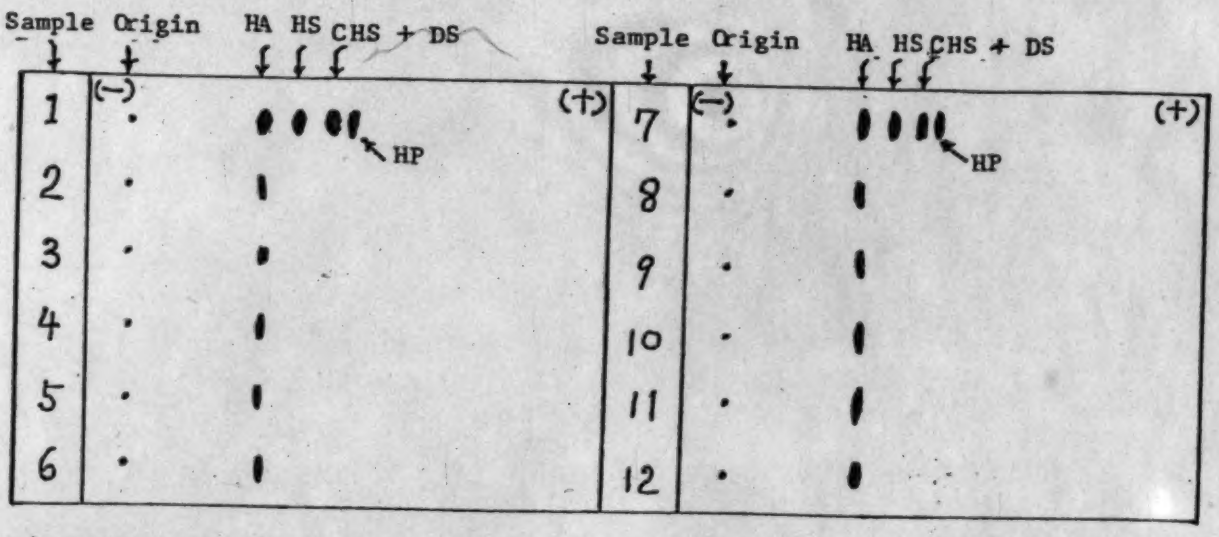
(b) 1.2M NaCl Fraction

Chemical and electrophoretic analyses of the 1.2M NaCl GAG fraction indicated that it contains a considerable amount of DS and a relatively small amount of HS, ChS and HP.

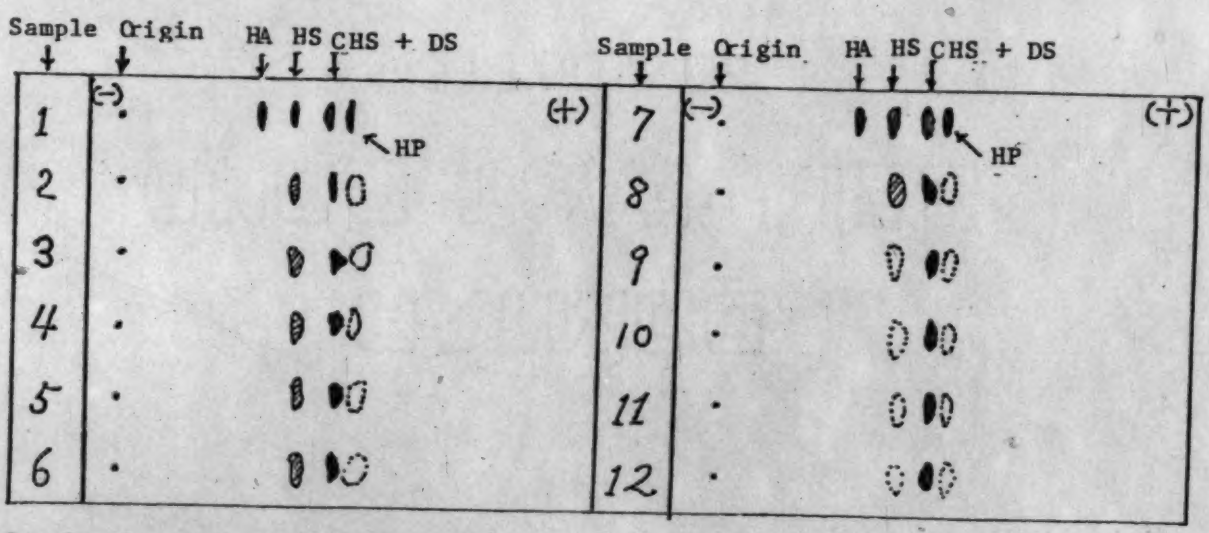
The Dische: Bitter and Muir uronic acid color yield ratio was lower than 1, 0.82 to 0.90 in intramuscular and 0.72 to 0.74 in epimysial tissues, and the carbazole (Dische) : orcinol uronic acid color yield ratio was lower than those obtained with standard Ch4-S and Ch6-S. Both of these observations suggest the presence of considerable amounts of DS. Testicular hyaluronidase susceptibilities were: 50% to 58% in the fetal intramuscular tissues, 41% to 47% in the post natal intramuscular tissues and 28% to 30% in the epimysial tissues. During gas liquid chromatographic analysis of the constituent hexosamines, galactosamine was detected with a small amount of glucosamine. The molar ratios of sulfate to hexosamine in this

FIGURE 12 Electrophoresis[1] of 0.4,1.2,and 2.1M NaCl Fractions on Cellulose Acetate Membranes.

a) 0.4M NaCl Fraction



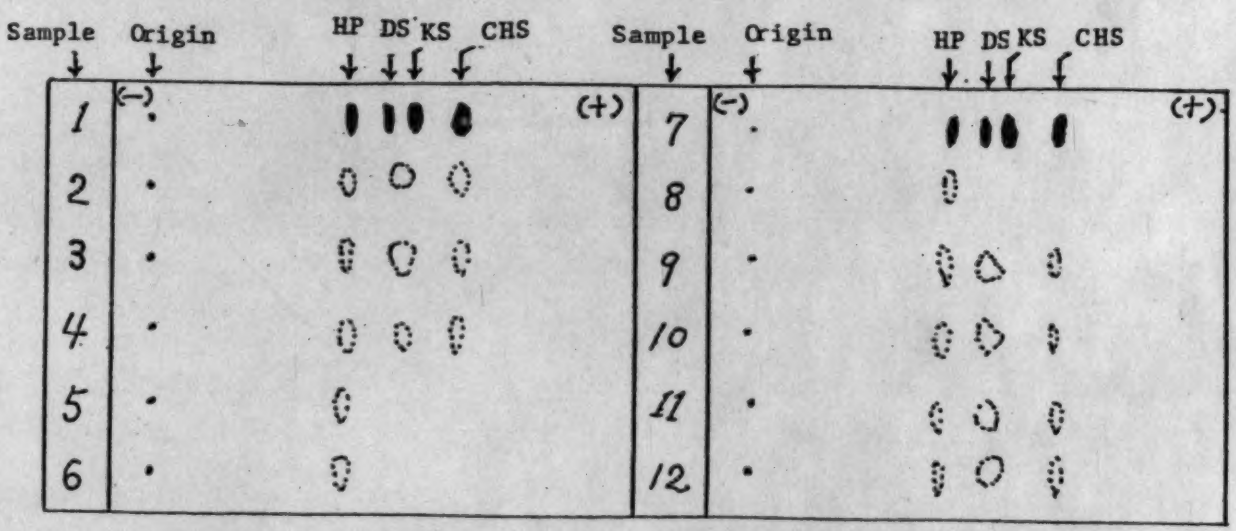

b) 1.2M NaCl Fraction




Sample: 1 and 7, mixture of standard GAG containing, HA, HS, CHS, DS and HP, 2,FI, 3,FII, 4,I, 5,IIf, 6,IIm, 8,III(2-6 and 8, intramuscular tissue), 9,I, 10,IIf, 11,IIm, 12,III(9-12, epimysial tissue).

FIGURE 12(continued)

c) 2.1M NaCl Fraction

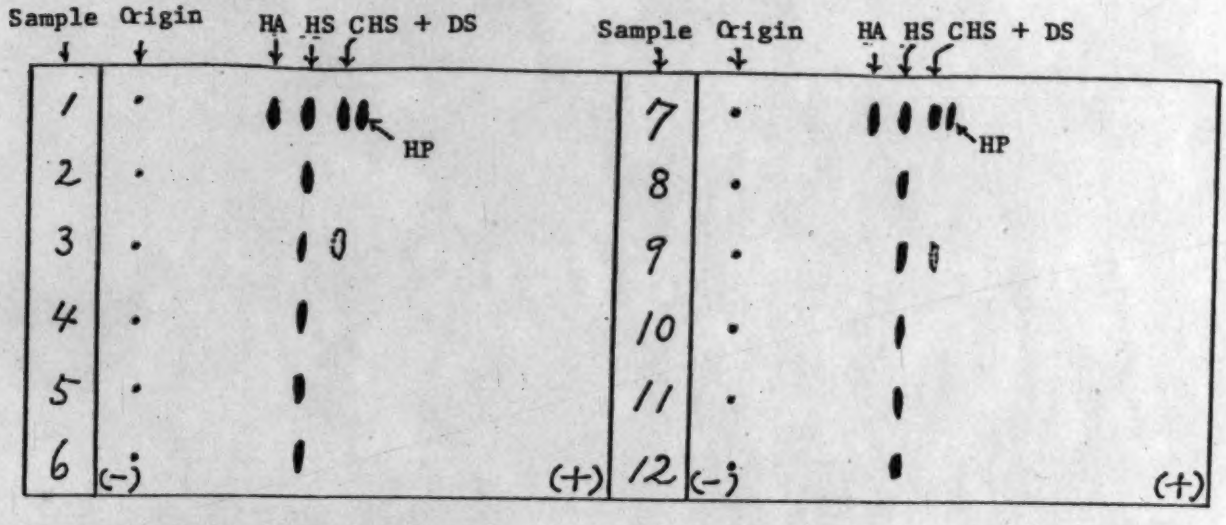



Sample: 1 and 7, mixture of standard GAG containing, HP, DS, KS, and CHS. 2,FI, 3,FII, 4,I, 5,IIf, 6,IIm, 8,III(2-6 and 8, intramuscular tissue), 9,I, 10,IIf, 11,IIm, 12,III(9-12, epimysial tissue).

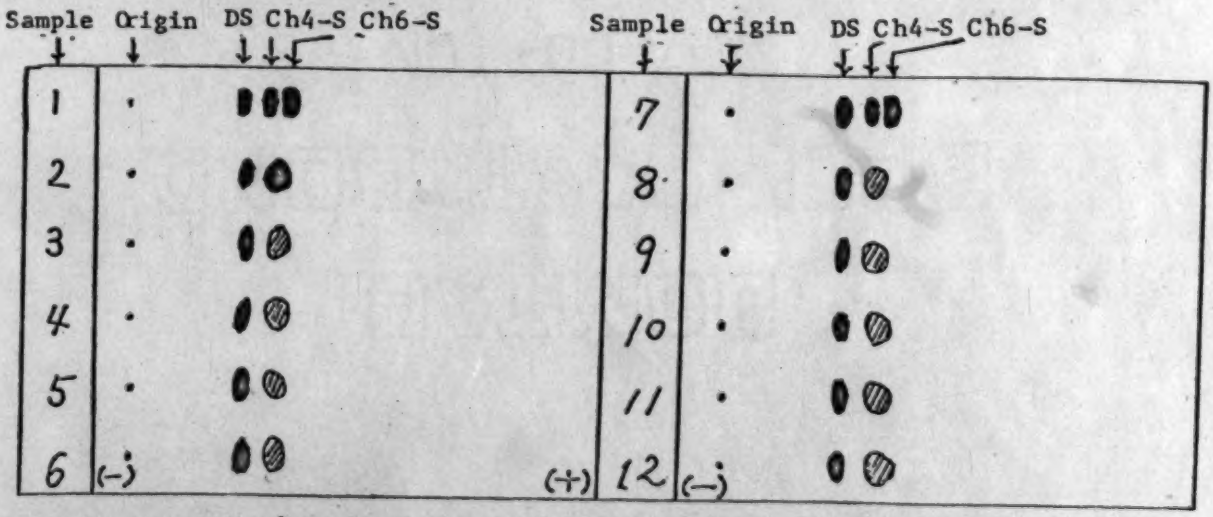
1 a) and b) in 0.1M phosphate buffer, and c) in 0.1M barium acetate solution.

FIGURE 13 Electrophoresis[1] of 1.25 and 1.5M NaCl Fractions From Dowex 1-X2 Column on Cellulose Acetate Membranes.

a) 1.25M NaCl Fraction




b) 1.5M NaCl Fraction




Sample: 1 and 7 mixture of standard GAG containing, HA,HS,CHS,DS and HP in a) and DS,Ch4-S, and Ch6-S in b) 2,FI, 3,FII, 4,I, 5,IIf, 6,IIm, 8,III(2-6 and 8, intramuscular tissue), 9,I, 10,IIf, 11,IIm, 12,III, (9-12, epimysial tissue).

1 a) in 0.1M phosphate buffer, and b) in 0.3M calcium acetate solution.

fraction were found to be in the range of 0.90 to 1.20, more or less similar to those of reference Ch4-S (0.97), Ch6-S (0.98) and HS (0.99) (Table 18).

Electrophoresis of the 1.2M NaCl fraction in 0.1M phosphate buffer and subsequent staining revealed three bands for all intramuscular and epimysial tissues studied (Figure 12b). The three bands had Rf values identical to those of standard DS, HS and HP. The DS band Rf was, however, identical to that of ChS in a 0.1M phosphate buffer. Further fractionation followed by additional characterization was deemed necessary to clearly identify the GAG composition of this fraction.

The 1.2M NaCl fraction accounted for 38 to 49% of the total GAG in the intramuscular tissues and 58 to 66% of the total GAG in the epimysial tissues, according to the hexosamine concentration values reported in Table 14.

(c) Further Fractionation and Characterization of the 1.2M NaCl GAG Fraction

a. Fractionation on a Dowex 1-X2 Column. Further fractionation of the 1.2M NaCl GAG fraction was conducted on a Dowex 1-X2 column. The GAG were eluted stepwise with increasing concentrations of NaCl starting with 0.5M. Ten ml fractions were collected and assayed for uronic acid. When the effluent gave a negligible uronic acid result, the concentration of NaCl was increased to 1.25M, followed by subsequent increases to and elutions with 1.5M and 2.0M NaCl.

As shown in Table 19, 91 to 97% of the uronic acid eluted was collected in the 1.25M and 1.5M NaCl fractions, which were further subjected to detailed chemical characterization procedures. The amounts of uronic acid in the 1.5M NaCl fractions were found to be much higher than those in the 1.25M NaCl fraction. The proportion of the 1.25M NaCl fraction in the intramuscular tissue was about two times greater than that in epimysial tissue (26 to 38% versus 10 to 18%). The color yield is not equivalent for each type of GAG when measured by means of the carbazole reaction; consequently each percentage shown in Table 19 does not necessarily represent the precise amount of GAG present.

b. Electrophoretic and Enzymatic Characterization

The 0.5M and 2.0M NaCl fractions containing trace amounts of GAG (see Table 19) were not characterized. The 1.25M NaCl fraction was subjected only to electrophoretic characterization, while the 1.5M NaCl fraction was subjected to both electrophoretic and enzymatic characterization.

Electrophoresis in 0.1M phosphate buffer and subsequent staining of all intramuscular and epimysial 1.25M NaCl fractions revealed a discrete band with an Rf value identical to that of the HS standard (Figure 13a). Two samples, however, showed a faint band with an Rf value identical to that of ChS and DS.

Electrophoresis of the 1.5M NaCl fraction in 0.3M calcium acetate solution and subsequent staining revealed two bands for all intra-

Table 19. Fractionation of the 1.2M NaCl Fraction on a Dowex 1-X2 Column

Each fraction collected was quantitatively analyzed as uronic acid and the proportion of each fraction was calculated as a percentage based on recovered amounts of uronic acid.

| Age and Sex Groups | Proportion (%) of Each Fraction | | | | % recovery of Uronic acid[1] |
|---|---|---|---|---|---|
| | 0.5M | 1.25M | 1.5M | 2.0M | |
| | | | Intramuscular Tissue | | |
| FI | 4 | 30 | 64 | 2 | 97.9 |
| FII | 6 | 31 | 61 | 2 | 95.9 |
| I | 4 | 37 | 56 | 3 | 90.6 |
| IIf | 6 | 26 | 67 | 1 | 105.0 |
| IIm | 5 | 38 | 56 | 1 | 94.4 |
| III | 4 | 30 | 64 | 2 | 97.9 |
| | | | Epimysial Tissue | | |
| I | 2 | 14 | 83 | 1 | 95.8 |
| IIf | 2 | 18 | 77 | 3 | 90.6 |
| IIm | 1 | 13 | 82 | 4 | 98.5 |
| III | 1 | 10 | 81 | 8 | 97.4 |

[1] Recovery was calculated on the basis of total uronic acid added to the column.

muscular and epimysial tissues studied (Figure 13b). The first and major band had an Rf value identical to that of the DS standard. The second band, which was relatively faint and broad, was likely composed of unseparated bands of Ch4-S and Ch6-S. It appeared difficult to get separation of these two GAG from muscular preparations by this electrophoretic technique, although the standard ChS isomers showed clearly separated bands (Figure 13b). Further, in the above broad band of ChS, the area corresponding to Ch4-S standard was found to be larger than that corresponding to Ch6-S standard.

The amount of GAG in each band was estimated colorimetrically by dissolving the dye bound GAG on the electrophoretogram in an aqueous solution of 5% CPC (Table 20). Enzymatic analysis of the 1.5M NaCl fraction was also carried out at the same time (Table 21). Both methods demonstrated a similar relative proportion of DS to ChS. These findings indicate that DS is the major galactosaminoglycan in the 1.5M NaCl fraction and accounts for 56% to 79% of the fetal intramuscular tissue, 71% to 81% of the post natal intramuscular tissue, and 80% to 87% of the epimysial tissue galactosaminoglycans. The younger fetal group had a considerably lower proportion of intramuscular DS (56% to 63%) than the older fetal group (70% to 79%) (Tables 20 and 21). This observation was consistent with that found previously in Analysis I. The enzymatic analysis of this fraction also gave a similar proportion of Ch4-S : DS : Ch6-S obtained in the 1.2M NaCl fraction (Analysis I) (Table 12). Therefore DS is the major galactosaminoglycan present in the bovine TB muscle of the animals studied ranging in age from 6 months pre partum to 8.9 years of age.

Table 20. Proportion of Isomeric Galactosaminoglycans (Ch4-S + Ch6-S:DS) Determined by the Colorimetric Measurement of the Stained Electrophoretogram.

| Age and Sex Groups | Proportion of Galactosaminoglycans Ch4-S + Ch6-S | DS |
|---|---|---|
| | Intramuscular Tissue | |
| FI | 44 (%) | 56 (%) |
| FII | 30 | 70 |
| I | 28 | 72 |
| IIf | 24 | 76 |
| IIm | 29 | 71 |
| III | 26 | 74 |
| | Epimysial Tissue | |
| I | 19 | 81 |
| IIf | 17 | 83 |
| IIm | 17 | 83 |
| III | 20 | 80 |

Table 21. Proportion of Isomeric Galactosaminoglycans (Ch4-S:DS:Ch6-S) by Enzymatic Methods.

| Age and Sex Groups | Proportion of Galactosaminoglycans | | |
|---|---|---|---|
| | Ch4-S | DS | Ch6-S |
| | | Intramuscular Tissue | |
| FI | 14 (%) | 63 (%) | 23 (%) |
| FII | 9 | 79 | 12 |
| I | 8 | 75 | 17 |
| IIf | 8 | 81 | 11 |
| IIm | 6 | 80 | 14 |
| III | 5 | 76 | 19 |
| | | Epimysial Tissue | |
| I | 2 | 87 | 11 |
| IIf | 4 | 82 | 14 |
| IIm | 7 | 83 | 10 |
| III | 5 | 81 | 14 |

(d) 2.1M NaCl Fraction Electrophoresis of the 2.1M NaCl GAG fraction suggested that it contained HP, DS, ChS and trace amounts of KS. Electrophoresis in 0.1M barium acetate solution and subsequent staining of this fraction gave three bands for some of the intramuscular and all of the epimysial tissues studied (Figure 12c). The first band had an Rf value identical to the HP standard. The second band was found to be broad with an Rf value similar to that of the DS standard. But this faster moving band appeared to slightly overlap another GAG with an Rf value similar to the KS standard. This may suggest the presence of negligible amounts of KS in this fraction; however, this point was not studied in further detail. The third band had an Rf value similar to that of the ChS standard.

The 2.1M fraction accounted only for 3% to 9% of the total intramuscular GAG and 2% to 3% of the total epimysial GAG pools according to the hexosamine concentration values reported in Table 14.

H. Protein Analysis

Protein analysis was conducted on all fractions because it is essential that GAG fractions contain as little protein as possible so as not to interfere with analysis (Anno, 1972).

Protein analysis of all fractions, except the 2.1M NaCl fraction, was performed by the method of Lowry et al. (1951). The 2.1M NaCl fraction was not analyzed because of its small size, 2 to 9% of total GAG hexosamine (Table 14). The amount of protein present in each fraction was expressed as a weight ratio of protein to hexosamine. As

shown in Table 22, the ratio values obtained were: 0.08 to 0.18 in the 0.4M NaCl fractions and 0.05 to 0.10 in the 1.2M NaCl fractions from all intramuscular and epimysial tissues studied. The protein : hexosamine ratios found in the three reference GAG ranged from 0.002 to 0.05 (Table 22).

## 2. Discussion

### (i) Quantitative Analysis of GAG as Uronic Acid in Analysis I

Glycosaminoglycans were measured as uronic acid in Analysis I, in which a quantitative comparison was made without correcting for differences in uronic acid color yield as affected by the structure of the GAG. In Analysis II, GAG were measured both as hexosamine and as uronic acid. Similar overall age and sex related total GAG patterns were observed in both Analyses I and II. Uronic acid values obtained in Analysis I therefore appear to be a meaningful indicator of total tissue GAG concentrations. The observations made in Analysis I regarding age and sex associated changes in GAG will be discussed later with the corresponding observations in Analysis II.

### (ii) Uronic Acid Test of the Non-GAG Fractions

Uronic acid analysis of the non-GAG fraction is not reported by many researchers (Pearson, personal communication). It was observed in Analysis II that the amount of GAG uronic acid present in the glycopeptide fraction and celite residue was negligible. However, the presence of uronic acid in the proteolytic enzyme undigested residue, which contained 1.4% to 8.7% of the total hexosamine (Table 13) was not tested in this study due to the difficulty in

Table 22. Protein/Hexosamine Ratio.

| Age and Sex Groups | Fraction: Lyophilized-defatted tissue | Filtrate | 0.4M NaCl | 1.2M NaCl |
|---|---|---|---|---|
| | Intramuscular Tissue | | | |
| FI | 218 | 0.68 | 0.16 | 0.08 |
| FII | 300 | 0.63 | 0.12 | 0.09 |
| I | 1038 | 0.69 | 0.13 | 0.08 |
| IIf | 874 | 0.63 | 0.10 | 0.05 |
| IIm | 1062 | 0.68 | 0.08 | 0.07 |
| III | 843 | 0.60 | 0.14 | 0.08 |
| | Epimysial Tissue | | | |
| I | 334 | 0.63 | 0.09 | 0.05 |
| IIf | 348 | 0.59 | 0.16 | 0.08 |
| IIm | 440 | 0.60 | 0.18 | 0.10 |
| III | 402 | 0.65 | 0.16 | 0.05 |
| | Reference GAG[2] | | | |
| | HA | 0.03 | | |
| | CHS | 0.002 | | |
| | HS | 0.05 | | |

[1]Weight ratio. Values of total hexosamine released were used in the case of lyophilized-defatted tissue.

[2]CHS: Sigma Chemical Co. and Others: gifts from Dr. Mathews.

analyzing this crude preparation. It has been reported that some of the tissue KS is not precipitated by the CPC method (Rodén et al. 1972). The presence of unprecipitable KS was not checked in this study. It was also suspected that smaller molecular weight portions of HP (<12,000)[1] may be lost through the dialysis bag; however, no attempt was made to verify this. Yoshizawa (personal communication) suggested that dialysis depends on the charge and form of molecules; thus smaller molecules do not necessarily pass through the wall of the dialysis bag.

(iii) Percentage Hexosamine Released During Preparation of a Sample for Fractionation by the CPC Method

The percentage hexosamine released from tissues by proteolytic digestion averaged 95.5% (Table 13) and is close to the value reported by Fox (1968), who digested bovine intramuscular tissue with alkali and pronase and found that 96% of the total hexosamine was released. The author has not found information in the literature concerning hexosamine released during epimysial tissue digestion.

The percentage of the total hexosamine in the glycopeptide fraction in this study (63.3%) was approximately two times higher than the 37% reported by Fox (1968). Fox (1968) however separated glycopeptide from GAG by means of ion exchange chromatography and did not present evidence demonstrating separation of the glycopeptide fraction from the GAG fraction. The use of an ion exchange column

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[1] Molecular weight of HP varies from 6,000 to 20,000 (Merck Index, 1968).

for separation of glycopeptides from GAG is not commonly practised. Recently, Vandenburgh et al. (1974) reported the presence of hexosamine in rat muscle sarcolemma. Possibly the sarcolemma accounts for the higher proportion of total hexosamine in the intramuscular glycopeptide fraction observed in this study. Information for epimysial glycopeptide is not available in the literature to the best of the author's knowledge.

(iv) Glycosaminoglycan Recovery after Fractionation by the CPC Method

The percentages of total GAG hexosamine obtained in this study were compared with those of Fox (1958) who reported recovery values for hexosamine from intramuscular tissue and of Cormier et al. (1971) who reported recovery values for hexosamine from epimysial tissue. Fox (1968) reported the proportion of the total intramuscular hexosamine in the GAG fraction to average 51.7%, or about 2 fold greater than the average value of 21.5% (Table 14) recorded in this study. This higher value appears to be possibly due to the lower proportion of glycopeptide hexosamine obtained by this author. Cormier et al.(1971), working with bovine SM epimysial tissue, reported recovery values for GAG, isolated as a cetylpyridinium complex, of 20% and 49% as hexosamine and uronic acid respectively. Their GAG hexosamine recovery appears to be less than one-half that found in this study, which averaged 49% (Table 14). Although the calculation basis is different in each study, i.e., total tissue hexosamine (Cormier et al. 1971) versus total hexosamine released, the recovery reported by these authors is undoubtedly low. These authors analyzed hexosamine and uronic acid

by the method of Boas (1953), a modified Elson-Morgan reaction, and Dische (1947), a carbazole reaction, respectively. However, it was not indicated how interfering non-hexosamine or non-uronic acid chromogens were removed from the crude samples. It is impossible to do a direct uronic acid assay on a crude sample (Davidson; Suzuki, both personal communications). In this study the crude samples were purified by means of the CPC method and column chromatography.

A small amount of GAG hexosamine in the 2.1M NaCl fraction (2% to 9%) on a total tissue GAG basis was observed in this study. Gilbreath et al. (1971), using the same CPC method for bovine intramuscular GAG separation, ignored the 2.1M NaCl fraction due to a lack of a positive uronic acid test.

(v) Concentration of Total Tissue GAG

Some inconsistency as to the total GAG concentration exists among values present in the literature (see Table 1), with those as hexosamine (Table 14) and uronic acid (Tables 11 and 15) obtained in this study. Gilbreath et al. (1971) reported total intramuscular GAG hexosamine concentrations in corresponding muscle and age groups to be less than one half of those reported in this study, (e.g., 96.6 µg per g, versus 223 µg per g in yearlings on a lyophilized-defatted basis). The basis for this difference cannot be readily determined at this time. To the best of the author's knowledge, information for intramuscular GAG measured as uronic acid is not available in the literature.

Cormier et al. (1971) reported total bovine SM epimysial GAG concentrations both as hexosamine and as uronic acid. However, their values are less than one half of those found for the corresponding post natal groups in this study (408 to 534 µg per g as hexosamine and 371 to 648 µg per g as uronic acid versus 1057 to 1341 µg per g as hexosamine and 1276 to 1735 µg per g as uronic acid in Analysis I and 1212 to 1571 µg per g as uronic acid in Analysis II on a lyophilized-defatted tissue basis. This difference may be due to the different muscle studied (SM) or may be due to differences in experimental technique. With regard to the latter possibility, no concrete suggestions can be made at this time.

The higher intramuscular GAG concentration found in the fetuses than in the post natal animals is likely due to the relatively smaller amount of intracellular material found in fetal muscle because of the smaller muscle fibers.

(vi) Relative Proportions of GAG in the 0.4M and 1.2M NaCl Fractions

The ratios of the amount of hexosamine in the intramuscular 0.4M : 1.2M NaCl fractions ranged from 1.42 to 1.51. Gilbreath et al. (1971) using the CPC method reported lower bovine TB intramuscular 0.4M NaCl : 1.2M NaCl ratios, for a 15 month old heifer (1.09) and higher ratios for a 9 year old cow (1.67) than those found from comparable age groups in this study. However, it is difficult at this time to suggest the reason for the above differences.

To the best of the author's knowledge, no information is

available in the literature for the value of the 0.4M NaCl : 1.2 M NaCl ratio in epimysial tissue.

(vii) Effect of Age and Sex on Bovine TB Muscle GAG, Glycopeptide and Sialic Acid Concentrations and Ratio of Total GAG to Collagen

The relatively constant post natal intramuscular total GAG concentration in both Analyses I and II is consistent with the report of Lagier and Exer (1960) cited by Sobel (1967). These authors demonstrated that the GAG concentration in human skin decreases in individuals up to 10 years of age and thereafter remains constant for at least 60 years in healthy individuals. Similarly, Gilbreath et al. (1971) studied bovine TB intramuscular tissue from a 3 month old calf, a 15 month old heifer, and a 9 year old cow, and found that the total GAG concentration was higher in the calf than in the heifer and old cow, which had similar concentrations. A gradual age related decrease of total epimysial GAG concentrations in both Analyses I and II was observed. Similarly, Cormier et al. (1971) studying bovine SM epimysial tissue from new born to 10.5 year old animals reported lower concentrations of GAG with increasing animal age. A decrease in the GAG concentration with increasing age has been reported in other tissues such as human aorta, myocardium and skin (Clausen, 1963), human heart valve (Torii et al., 1965), human rib cartilage (Iwata, 1969, cited by Tominaga et al., 1973), and in chicken skin (Kondo et al., 1971). To the best of this author's knowledge, information regarding fetal muscle GAG concentrations is not available in the present literature.

The increased proportion of DS during fetal growth (Tables 12, 20 and 21) may be of physiological significance to the intramuscular connective tissue ground substance during this period.

The values for intramuscular and epimysial glycopeptide concentrations reported in this study probably are the first reported for skeletal muscle.

The concentration of GAG (Tables 11, 14 and 15) tended to be lower in young adult males than in young adult females, although there were no statistically significant differences found between sex groups in Analysis I (Table 11). The concentrations of glycopeptide hexosamine (Table 14) and sialic acid (Table 16) were also found to be lower in tissues from the young adult males than from the young adult females. A significantly lower epimysial hexosamine concentration was found in the labile fraction obtained from the young adult males than from the young adult females (Table 9). Cormier et al. (1971) reported that the concentration of HA in bovine SM epimysial tissue is higher in heifers than in steers or aged cows. Gilbreath et al. (1971) have suggested that a higher intramuscular GAG concentration in a 9 year old cow than in a 15 month old heifer is possibly due to hormonal changes associated with lactation. Muscle from male animals tends to be relatively lower in ground substance components such as GAG and glycopeptide than muscle from female animals of a similar age.

The observed lower hexosamine : hydroxyproline (Figure 10) and uronic acid : hydroxyproline (Figure 11) ratios in young adult males

than in young adult females implies that connective tissue from male animals contains relatively less ground substance and more collagen than connective tissue from females of a similar age.

(viii) Characterization of Each GAG Fraction

Chemical and electrophoretic analyses of the bovine TB muscle GAG fraction revealed that HA and DS are the major GAG and that HS, Ch4-S, Ch6-S and HP are the minor GAG in both intramuscular and epimysial tissues from all animals studied. The relative proportion of each GAG was further estimated. The data used for this purpose are the observed proportions of GAG in the 0.4M and 1.2M NaCl fractions (Table 14), the proportions of the galactosaminoglycans (Tables 12, 20 and 21), the proportions of HS (1.25M NaCl fraction) : ChS + DS (1.5M NaCl fraction) (Table 19) and the color yield correction factor in the uronic acid carbazone reaction, 0.81 for DS and 1.30 for HS to 1.00 for ChS (Bitter and Muir, 1962). The estimated proportions are summarized in Table 23. However it has to be emphasized that these are estimated values and are presented to provide an overall description of the GAG composition of intramuscular and epimysial tissues.

In the intramuscular tissue, the relative proportion, HA : DS is approximately 48 : 24 in the younger fetal group and approximately 54: 23 in the older fetal and all post natal groups. While in the epimysial tissue the proportion is approximately 32:49 in the yearling and young male and female adult groups and approximately 42 : 42 in the mature adult group. Other GAG

Table 23. Estimated Proportion of Bovine TB Muscle GAG

| Age and Sex Groups | GAG | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HA | DS | (HA+DS) | Ch4-S | Ch6-S | HS | HP | Total |
| | | | | Intramuscular Tissue | | | | |
| FI | 48[1] | 24 | (72) | 5 | 9 | 12 | -[2] | 98 |
| FII | 57 | 23 | (80) | 3 | 4 | 10 | - | 97 |
| I | 53 | 20 | (73) | 2 | 5 | 11 | - | 91 |
| IIf | 52 | 25 | (77) | 2 | 3 | 8 | - | 90 |
| IIm | 51 | 23 | (74) | 2 | 4 | 13 | - | 93 |
| III | 54 | 22 | (76) | 1 | 6 | 9 | - | 92 |
| | | | | Epimysial Tissue | | | | |
| I | 32 | 51 | (83) | 1 | 7 | 7 | - | 98 |
| IIf | 31 | 47 | (78) | 2 | 8 | 9 | - | 97 |
| IIm | 31 | 50 | (81) | 4 | 6 | 6 | - | 97 |
| III | 42 | 42 | (84) | 3 | 7 | 4 | - | 98 |

[1]% of Total GAG, calculation based on hexosamine concentration.

[2]Trace.

proportions are also shown in Table 23.

McIntosh (1966) reported ChS to be the only GAG present in bovine intramuscular tissue. After that, Fox (1968) reported the presence of HA, Ch and ChS in bovine intramuscular tissue; Wipf (1969) and Wipf et al. (1970) reported the presence of HA, Ch, ChS, DS and HP in porcine intramuscular tissue, and Gilbreath et al. (1971) reported the presence of HA and ChS (or DS) in bovine intramuscular tissue. These authors, however, did not thoroughly characterize these GAG. Cormier et al.(1971) determined the presence of HA, DS and ChS in bovine epimysial tissue by electrophoretic techniques. Comparing with these intramuscular and epimysial GAG reported above, all the GAG found in this study were more extensively characterized. Fox (1969), Wipf (1969) and Wipf et al. (1970) reported the elution of Ch with 1.25M NaCl on a Sephadex A-25 ion exchange column without characterization data. Small amounts of galactosamine, the constituent hexosamine in the galactosaminoglycans, observed in the 0.4M NaCl fractions (Table 17). might be indicative of the presence of Ch; however no Ch like spot was detected by electrophoresis. The presence of Ch has been conclusively reported in a limited number of tissues such as the cornea (Davidson and Meyer, 1954) and the skin of the squid (Anno et al., 1964) and octopus (Seno et al., 1965). Wipf (1969) digested porcine intramuscular tissue with alkali and pronase. These authors reported that an insoluble portion remaining after digestion was exclusively DS; however no data conclusively identifying this GAG was provided. In this study DS was found to be a major galactosaminoglycan in the epimysial tissue. This

observation is consistent with the findings of Cormier et al. (1971) who reported that the main GAG in bovine epimysial tissue eluted with 1.5M NaCl on Dowex 1-X2 column was DS. The data reported herein for the isomeric chondroitin sulfates are probably the first for the separation of Ch4-S and Ch6-S in either intramuscular or epimysial tissue. This is also probably the first report for identification and characterization of HS and HP in either tissue. The presence of HP was reported by Wipf (1969) and Wipf et al. (1970); however no characterization data was presented.

Electrophoresis gave an incompletely separated band of Ch4-S and Ch6-S (Figures 13b), where the area corresponding to the Ch4-S standard was found to be larger than that corresponding to the Ch6-S standard. This suggests that the proportion of Ch6-S is lower than that of Ch4-S, contrary to the result obtained by the enzymatic method (Tables 11 and 21). However, a similar observation has been reported by Kondo et al. (1971) who separated chicken skin galactosaminoglycans using electrophoretic and enzymatic methods and found that Ch6-S was hardly detectable by electrophoresis, while enzymatic methods indicated that Ch6-S accounted for approximately 10% of the fraction. The same authors suggested the presence of a heterogeneous structure in both the Ch4-S and the DS molecules with regard to the position of sulfate groups and uronic acid residues. Recent literature indicates some heterogeneous structures of GAG (see Review of Literature). Possibly bovine TB muscle galactosaminoglycans are of a heterogeneous structure.

(ix) Protein Analysis

The slightly higher protein : hexosamine ratios in the 0.4M NaCl fraction than in the 1.2M NaCl fraction (Table 22) were possibly due to contamination by the glycopeptide fraction which may not have been completely dissolved by the 0.4M NaCl washing.

Fox (1968) reported extremely high nitrogen : hexosamine ratios of 56.7, 85.6, 18.5 and 6.1 in his respective 0.05, 0.5, 1.25 and 1.5M NaCl fractions obtained by ion exchange chromatography. Schiller et al. (1954) found the nitrogen : hexosamine ratio to be 0.8 in the supernatant following precipitation with TCA, and 0.14 in ethanol precipitated GAG. Kondo et al. (1971) reported the ratio of protein analyzed by Lowry et al. (1951) to hexosamine to be 0.12 to 0.35 in the ethanol precipitated sodium salt of whole GAG obtained from chicken skin. Therefore the results for the protein : hexosamine ratios reported in this thesis for the isolated GAG fractions compare favourably with those reported by other authors.

# GENERAL CONCLUSIONS

A study was designed to determine the effect of age and sex on the bovine TB intramuscular and epimysial connective tissue components; collagen, elastin, reticulin, GAG, glycopeptide, and sialic acid. Particular emphasis was placed on the GAG.

Under the conditions of this study, the main observations obtained were as follows:

1) Histochemical observations showed that fetal intramuscular collagen scores were significantly higher than post natal collagen scores. In contrast, fetal epimysial collagen scores were significantly lower than post natal collagen scores. The intramuscular collagen score did not change during fetal growth, while the epimysial value decreased significantly during this growth period. No significant difference in the collagen score was found in either intramuscular or epimysial tissues from the post natal groups.

Both intramuscular and epimysial reticulin scores were significantly higher in the fetal than in the yearling groups, which in turn had a significantly higher score than the adult groups. The reticulin scores for all adult groups were similar. Endomysial tissue displayed a reticulin positive response in all intramuscular tissues studied.

The collagen score : reticulin score ratios, an estimate of the relative amount of these two connective tissue proteins, were found to be significantly higher in the fetal than in the post natal groups. The intramuscular ratios were similar among all post natal groups, while the epimysial ratios increased significantly with increasing animal age.

A relatively limited, though constant amount of elastin was found in both intramuscular and epimysial tissues. The elastin was found largely in the blood vessels of the perimysial and epimysial tissues.

The MPS scores of the younger fetal groups were significantly higher than those of the older fetal groups for both intramuscular and epimysial tissues. These fetal MPS scores were in turn significantly higher than those of all post natal MPS scores. Within the post natal groups, there was a gradual decline in the MPS score with increasing animal age.

The intramuscular and epimysial MPS score : collagen score ratios, an estimate of the relative amount of ground substance to collagen, decreased significantly during fetal growth. The ratios were significantly higher in the fetal than in the post natal groups. In the post natal groups, the intramuscular ratio did not change significantly, whereas the epimysial ratio was significantly lower in the mature adults than in the yearlings.

The histochemical observations were consistent with the chemical observations reported in this thesis.

2) Intramuscular nitrogen concentrations were found to be lowest in the younger fetal group, and to significantly increase during fetal and early post natal growth. The intramuscular and epimysial nitrogen concentrations were similar in all post natal groups averaging 154.5 mg per g and 175.4 mg per g lyophilized-defatted tissue respectively. The post natal nitrogen concentrations of the epimysial tissues were approximately 13% greater than those of the intramuscular tissues.

Intramuscular collagen concentrations, in contrast to the nitrogen concentrations, were highest in the youngest fetal group and decreased significantly during fetal and early post natal growth. The intramuscular collagen concentrations were similar in all adult groups (average 32.0 mg per g lyophilized-defatted tissue), while the epimysial collagen concentrations were significantly higher in the mature adult group than in the younger post natal groups, and were also significantly higher in the young male adult group than in the young female adult group of Trial I animals. The collagen concentrations of the epimysial tissues were approximately 24 fold greater than those of the intramuscular tissues from animals of comparable ages.

3) The percentage of the collagen that was labile (solubilized by heating in a 0.15M NaCl solution at 77 C for 1 h) decreased significantly with advancing animal age in both intramuscular and epimysial tissues from all animals studied. The rate of decrease was found to be slower in the epimysial than in the intramuscular tissues.

The intramuscular labile hexosamine concentrations were significantly higher in the fetal than in the post natal groups and decreased significantly during fetal growth. There were no significant differences found in the intramuscular labile hexosamine concentrations in the post natal groups. The epimysial labile hexosamine concentrations were significantly lower in the mature adult group than in the yearling and young female groups, and were significantly lower in the male adult than in the young female adult

group.

The intramuscular labile salic acid concentration was found to decrease significantly during fetal growth. In the post natal animals, negligible amounts ( <0.2 mg per g lyophilized-defatted tissue) of labile sialic acid were found in both the intramuscular and epimysial tissues.

The intramuscular SNSPN concentration decreased significantly during fetal growth. Both fetal groups contained a significantly greater amount of intramuscular SNSPN than did the post natal groups. A slight decrease in intramuscular SNSPN was observed during post natal growth. The epimysial SNSPN concentration was observed to be significantly lower in the mature adult group than in the other post natal groups.

4) Glycosaminoglycans were determined as hexosamine and uronic acid. Hexosamine recovery was determined during the isolation and fractionation of GAG. An average of 95% of the hexosamine was liberated into the effluent during tissue proteolysis. The intramuscular and epimysial GAG fractions were found to contain 22% and 49% of the hexosamine respectively.

A relatively higher proportion of hexosamine was found in the intramuscular glycopeptide fraction (average 70%) than in the epimysial glycopeptide fraction (average 43%).

Glycosaminoglycan concentrations were measured as uronic acid in the 0.4M, 1.2M and 2.1M NaCl fractions obtained by the CPC method

in Analysis I. Total intramuscular uronic acid concentration decreased significantly during fetal growth, and was significantly higher in the fetal than in the post natal groups. This age related decrease was associated with a significant decrease of the total uronic acid to collagen ratio. There were no significant differences in the total intramuscular uronic acid concentrations (average 223 μg per g lyophilized-defatted tissue) among the post natal groups. Total epimysial uronic acid concentration decreased gradually and significantly with advancing age in the post natal groups. The epimysial total uronic acid to collagen ratio was significantly lower in the mature adult group than in the yearling and young female adult groups. This ratio was also significantly lower in the young male adult group than in the young female adult group suggesting that the male epimysium contains relatively less ground substance and more collagen than does the female epimysium.

The intramuscular uronic acid concentration decreased significantly in each of the three NaCl fractions during fetal growth. It was also significantly higher in the fetal than in the post natal groups. There were no significant differences in the intramuscular uronic acid concentrations among the post natal groups within each of the salt fractions. The epimysial uronic acid concentration decreased continually with increasing animal age in the 1.2M NaCl fraction with the mature adult group displaying a significantly lower concentration than the other post natal groups. There were no significant age related differences in the epimysial uronic acid concentrations

of the 0.4M and 2.1M NaCl fractions. Uronic acid concentrations in Analysis I were consistent with both the hexosamine and the uronic acid concentrations in Analysis II.

The largest GAG fraction in the intramuscular tissue was the 0.4M NaCl fraction, while the 1.2M NaCl fraction was the largest in the epimysial tissue, whether based on uronic acid or hexosamine concentrations. An overall comparison of the post natal groups revealed that the epimysial tissues contained a five to six fold greater concentration of total GAG than did the intramuscular tissues.

Chemical and electrophoretic analyses revealed that the 0.4M NaCl fraction was almost entirely composed of HA in both the intramuscular and epimysial tissues. The major intramuscular GAG in the 1.2M NaCl fraction was DS, accounting for 56% to 63% in the younger fetal group and 70% to 81% in the older fetal and post natal groups. The major epimysial GAG component in the 1.2M NaCl fraction was also DS, accounting for 80% to 87% of the total GAG in this fraction. The 2.1M NaCl fraction accounted for only 3% to 9% of the total intramuscular GAG and 2% to 3% of the total epimysial GAG pool based on a hexosamine concentration. Electrophoresis of this fraction suggested that it contained HP, DS, ChS and trace amount of KS.

It was concluded that HA and DS are the major GAG in both the intramuscular and epimysial tissues from animals ranging in age from 6 months prepartum to 8.9 years of age. The concentration of HA was approximately twice that of DS in the intramuscular tissues, while only approximately 60% that of DS in the yearling and young adult

epimysial tissues. The mature adult epimysium contained almost equimolar amounts of HA and DS. Relatively small amount of HS, Ch4-S, Ch6-S, and HP were also found in all tissues.

5) The concentrations of sialic acid and of glycopeptide, measured as hexosamine, decreased during fetal growth, and remained relatively constant among the post natal groups in the intramuscular tissue (184 to 193 μg/g sialic acid and 640 to 762 μg/g hexosamine on a lyophilized-defatted basis). However they were found to decrease gradually with increasing animal age in the epimysial tissue.

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# APPENDIX I

## Preliminary Treatment of Dowex 1-X2 Resin

Preliminary treatment of Dowex 1-X2 resin was carried out as follows. Uniform size resin was collected by soaking in distilled water. The resin was then washed with 1N NaOH by stirring it in a beaker for about 5 min, followed by filtration using a Buchner funnel, and was washed with distilled water until the filtrate became neutral. The resin was here converted to $OH^-$ form. This was washed with 1N HCl by stirring it in a beaker about 5 min, followed by washing with distilled water until the filtrate became neutral. These series of washings with the NaOH, water, HCl and water were repeated several times. Finally the $OH^-$ form was converted to $Cl^-$ form by washing with 1N HCl. A slurry of this resin was packed into a column (0.9 x 44 cm) and washed with distilled water until $Cl^-$ ion could not be detected with 1% $AgNO_3$ in the washings.